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(54) Title: 12(S)–HETE RECEPTOR BLOCKERS

(57) Abstract

The 12–lipoxygenase product, 12(S)–HETE, mediates hyperproliferative and hyperplastic responses seen in atherosclerosis, diabetes, Parkinson's disease, Alzheimer's, stroke–induced nerve damage and cancer. 12–HETE also mediates inflammation and cell death in some cell systems, particularly B–islet cells of the pancreas. The present invention involves amelioration of disease states mediated by 12(S)–HETE by blocking specific 12(S)–HETE receptors.

12(S)-HETE RECEPTOR BLOCKERS

This application claims priority from provisional
application 60/062,335, filed October 15, 1997.

STATEMENT REGARDING FEDERALLY SUPPORTED RESEARCH

5   This invention was made with government support
under Grant No. DK 39721 awarded by the National
Institutes of Health (NIDDK). The government may have
certain rights in the invention.

BACKGROUND OF THE INVENTION

10   ### Field of the Invention

The present invention relates to blockade of the
12(S)-HETE cell surface receptor as a treatment for
conditions of the body which result from stimulation or
overstimulation of the receptor. 12(S)-HETE, a product
15   of the 12-lipoxygenase pathway, mediates the
hyperproliferative and inflammatory responses present
in such diseases as atherosclerosis, psoriasis,
diabetes, and cancer. 12(S)-HETE also mediates
inflammatory responses and cell death in some cell
20   types, particularly pancreatic islet beta cells and
nerve cells. Blockade of the 12(S)-HETE receptor

ameliorates the symptoms and arrests the mitogenic

cellular responses.

Background

Lipoxygenases (LO) are metabolic enzymes which

5   catalyze the stereospecific oxygenation of

polyunsaturated fatty acids to hydroperoxy fatty acids

(Hamberg et al., J. Biol. Chem. 242:5329-5335 (1967)).

The physiological function of 12-LO, the mammalian

enzyme which catalyzes the oxygenation of arachidonic

10 acid to (S)-12-hydroperoxyeicosatetraenoic acid (12-

HPETE) and (S)-12-hydroxyeicosatetraenoic acid (12(S)-

HETE), is unclear.

12-LO exists as two isoforms which are the

products of different genes, leukocyte-type 12-LO and

15 platelet-type 12-LO, which share 65% homology at the

amino acid level (Izumi et al., Proc. Natl. Acad. Sci.,

USA 87:7477-7481 (1990); Funk et al., Proc. Natl. Acad.

Sci. USA 87:5638-5642 (1990)). The products of the 12-

LO pathway, such as 12(S)-HETE, have been shown to play

20 important roles in diseases such as atherosclerosis,

diabetes, and cancer.

12(S)-HETE has direct mitogenic and hypertrophic

effects in vascular cells. It is also a potent

chemoattractant for vascular smooth muscle cells (VSMC) and can activate oncogenes such as c-fos and ras and key growth-related kinases such as mitogen-activated protein kinases (ERK, JNK, PAK, p38) and protein kinase C. New results also indicate that 12(S)-HETE can directly increase monocyte binding. Human aortic endothelial cells incubated with 12(S)-HETE for four hours prior to monocyte adhesion assays resulted in an average increase of 3-fold (range of 1.5-5 fold) in monocyte binding as compared to untreated cells. In addition, glucose-induced monocyte adhesion was abrogated by the inhibition of 12-LO using both phenidone, a non-specific LO inhibitor, and baicalein, a more specific 12-LO inhibitor. The adhesion caused by 12-LO products appears to be monocyte-specific.

The 12-LO pathway is activated in pancreatic islets by cytokines and may participate in islet cell destruction. In inflammatory diseases, this pathway plays crucial roles in transmitting distinctive signals within the cell. Using inhibitors of the 12-LO enzyme pathway, researchers have been able to prevent inflammation and cellular damage. Furthermore, VSMC cultured under high glucose (HG) conditions produce increased amounts of 12(S)-HETE (Natarajan et al.,

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Proc. Natl. Acad. Sci. USA 90:4947-4951 (1993). Thus,
this pathway may be key to the accelerated
cardiovascular disease observed in diabetes.

The LO pathway also plays a role in the growth-

5   promoting effects of angiotensin II (AII) and in the
chemotactic effects of platelet-derived growth factor:
the products of the 12-LO pathway, and 12(S)-HETE in
particular, are associated with the hypertrophic,
hyperplastic, and mitogenic effects induced by AII.

10   Wen _et al._, 271 _Am. J. Physiol._ (40 Cell Physiol.)
C1212-C1220 (1996); (Natarajan _et al._, _Hypertension_
23:I142-I147 (1994)). The proliferative effects of AII
are inhibited by baicalein, a LO inhibitor. The
mitogenic effects of 12(S)-HETE are similar to those of

15   AII and are abrogated by pertussis toxin, implicating a
G-protein mechanism. The 12-LO enzyme pathway is known
to generate proinflammatory mediators in a variety of
cells (O.R. Etingin _et al._, _J. Lipid Res._ 31:299-305
(1990); V.A. Folcik and M.K. Cathcart _J. Lipid Res._

20   34:69-79 (1993)). Human and rat pancreatic B-cells
specifically express active leukocyte type 12-LO (V.P.
Shannon _et al._, _Am. J. Physiol._ 263:E828-E836 (1992):
D.S. Bleich _et al._, _Endocrinol._ 136:5736-5744 (1995)).
Recent evidence implicates products of the 12-LO

pathway in nerve cell death associated with Parkinson's disease, Alzheimer's disease and other inflammatory nerve cell conditions (Neuron 19:453-463 (1997)).

Because 12(S)-HETE has several biological effects linked to cellular growth in vascular smooth muscle and cardiac fibroblasts (Natarajan et al., Hypertension 23:I142-I147 (1994); Wen et al., Am. J. Physiol. 211:C1212-C1220 (1996)), it is implicated in the etiology of cardiovascular disease. Further evidence that 12(S)-HETE is responsible for the cellular responses seen in cardiovascular disease in diabetic patients includes the fact that monocyte binding to cultured human aortic endothelial cells increases in chronic high glucose conditions, and that this is coincident with increased formation of LO products such as 12(S)-HETE. (Kim et al., Diabetes 43:1103-1107 (1994)). Furthermore, treatment of aortic endothelial cells with 12(S)-HETE increases monocyte binding, likely by stimulating JNK activity and inducing CS-1. 12(S)-HETE can also stimulate vascular endothelial growth factor (VEGF) gene expression in vascular smooth muscle (Am. J. Physiol. 273: H2224-H2231 (1997)). VEGF has been linked to angiogenesis in diabetic

retinopathy, tumor growth and atherosclerotic vascular disease.

12(S)-HETE is also regarded as a mediator of inflammation and hyperproliferation of the skin (Arenberger et al., Skin Pharmacol. 6:148-151 (1993); Gross et al., J. Invest. Dermatol. 94:446-451 (1990)) and is therefore implicated in skin diseases. 12(S)-HETE has been shown to enhance tumor cell adhesion to endothelial cells. (Honn et al., Cancer Metastasis Rev. 13:365-396 (1994)). 12(S)-HETE can directly increase p21 activated kinase (PAK). The effect appears to be through activation of small GTP binding proteins such as RAC and through activation of PI3K.

The precise mechanisms of 12(S)-HETE action are not clear, however recent studies have shown that the LO product, 12(S)-HETE, activates c-jun amino terminal kinase (JNK) (Wen et al., Circ. Res. 81:651-655 (1997)). JNK is a small GTP-binding protein and a member of the MAP kinase family which is involved in cellular growth, inflammation, and apoptosis (Force et al., Circ. Res. 78:947-953 (1994)) and in cell cycle progression through G_1 (Olson et al., Science 269:1270-1272 (1995)). Evidence shows that JNK can serve as a

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positive or negative modulator of cell growth in
different cells. Olson et al., 269 Science 1270-1272
(1995); Yan et al., 372 Nature 798-800 (1994). 12(S)-
HETE activation of JNK may also be the mediator of
5   cytokine-induced pancreatic B-cell damage (Bleich
et al., Biochem. Biophys. Res. Commun. 230:448-451
(1997)).

Newer evidence indicates that the growth factor
and potent vasoconstrictor AII, linked to type-1
10   receptor activation, can activate JNK and PAK (Wen et
al., Circ. Res. 81:651-655 (1997); Schmitz et al.,
Circ. Res. 82:1272-1278 (1998)). Furthermore, AII can
modulate serum deprivation-induced apoptosis by
increasing JNK activity in vascular smooth muscle
15   cells, Sueror et al., Circulation, Supp. 1, I-281
(1994), mediated by lipids derived from the 12-LO
pathway, such as 12(S)-HETE. This indicates that 12-LO
products participate in JNK activation at least in part
through G_1-protein signaling. The ability of pertussis
20   toxin to block the activation of JNK by 12(S)-HETE also
supports the theory that 12(S)-HETE is a mediator of
AII-induced JNK activation through a G_1-mediated
pathway.

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While several studies have demonstrated the potent
biological effects of lipoxygenase products, the
mechanisms of action of these effects are not known.
Some reports have hinted at the presence of 12(S)-HETE

5   receptors on transformed cells. Binding sites for
12(S)-HETE have been detected in carcinoma cells
(Herbertsson and Hammarstrom, FEBS 298:249-252 (1992),
on melanoma cells (Liu et al., Proc. Natl. Acad. Sci.
USA 92:9323-9327 (1995), and in a human epidermal cell

10   line (Gross et al., J. Invest. Dermatol. 94:446-451
(1990); Suss et al., Exptl. Cell Res. 191(2):204-208
(1990)).

The 12(S)-HETE receptors described in carcinoma
cells are cytosolic receptors (Herbertsson and

15   Hammarstrom, Biochem. Biophys. Acta 1244:191-197
(1995)), activation of which may mediate 12(S)-HETE
induced mRNA production of genes coding for the
integrin $\alpha_{IIb}\beta_3$ (Chang et al., Biochem. Biophys. Res.
Comm. 176:108-113 (1991)). The localization of this

20   receptor is different from plasma cell membrane
receptors coupled to a G-protein and acting through
second messengers.

12(S)-HETE receptors on the cell surface of murine
melanoma cells have been described. These receptors

stimulate the second messengers diacylglycerol and
inositol phosphate$_3$ via a G-protein mechanism, resulting
in protein kinase C$_2$ activation.   (Liu et al., Proc.
Natl. Acad. Sci. USA 92:9323-9327 (1995)). The binding

5   of 12(S)-HETE to these receptors was blocked by 13(s)-
hydroxyoctadecadienoic acid, a LO metabolite of
linoleic acid, ablating the 12(S)-HETE increased
adhesion of the cells to fibronectin. These authors
suggest 12(S)-HETE may act in a "cytokine" fashion to

10   regulate responses of adjacent tumor cells, endothelial
cells, and platelets.

Receptors for 15-HETE have been identified in
mast/basophil (PT-18) cells and were shown to possess
properties of G-protein-coupled receptors (Vonakis and

15   Vanderhoek, J. Biol. Chem. 267:23625-23631 (1992).
Specific binding of 15-HETE to these receptors
stimulated 5-LO, and while 12(S)-HETE was found to be
an effective competitor of [^{3}H]15-HETE binding to PT-18
cells, suggesting that 12(S)-HETE binds to the specific

20   15-HETE receptor, the binding of 12(S)-HETE did not
stimulate the lipoxygenase. Very recent studies have
indicated the activation of a cell surface G-protein-
coupled 5-HETE receptor in neutrophils (Capadici
et al., J. Clin. Invest. 102:165-175 (1998)).

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The high affinity 12(S)-HETE-specific receptors in a human epidermal carcinoma cell line were induced by γ-IFN (Gross et al., J. Invest. Dermatol. 94:446-451 (1990)). Saturation binding of 12(S)-HETE to these

5   receptors did not stimulate cell growth, therefore, the function of these receptors in the skin is entirely speculative, and not related to the AII-induced cellular effects mediated by cell surface 12(S)-HETE receptors in fibroblasts overexpressing the AII

10   receptor and potentially in vascular smooth muscle cells. Two recent studies have indicated two additional agents which could reduce 12(S)-HETE binding (Kemeny and Ruzicka, Agents Actions 32:339-342 (1991); Kemeny et al., Arch. Dermatol. Res. 283:333-336

15   (1991)).

Specific inhibitors of 12-LO have been described. Gorins et al., J. Med. Chem. 39:4871-4878 (1996). In that study, a series of substituted (carboxyalkyl)benzyl ethers were found to be selective

20   inhibitors of leukocyte-type 12-LO. These inhibitors of 12-LO acted by serving as structural analogs for the enzyme. Gorins et al., J. Med. Chem. 39:4871-4878 (1996). The 5-LO inhibitor, 2-phenylmethyl-1-naphthol (DuP654), has also been shown to specifically inhibit

binding of 12(S)-HETE to receptors on the human
epidermal cell line SCL-II. Arenberger et al., Skin
Pharmacol 6:148-151 1993).

In vivo, inhibition of 12-LO has lowered blood
pressure in several models of hypertensive animals,
including rats (Stern et al., Am. J. Physiol. 257:H434-
H443 (1989); Nozawa et al., Am. J. Physiol. 259:H1447-
H1780 (1990)). In addition, blockage of 12-LO activity
has alleviated the growth-factor induced effects of
12-HETE in vascular cells. This, along with the known
increased expression of 12-LO observed in animal models
of diabetes (Gu et al., Am. Diabet. Assoc. Meeting
(1996); Natarajan et al., Intl. Aldosterone Meeting
(1998)) and diabetes induced accelerated
atherosclerosis (Gerrity et al., Circulation I175
(1997)) strongly implicate 12-HETE and the 12-LO
pathway in the etiology of these diseases. The harmful
effects of 12-LO activation are ameliorated by blocking
the production of 12(S)-HETE, providing the rationale
for a method of treatment which focusses on preventing
12(S)-HETE binding to its receptor.

There is currently no inhibitor of 12(S)-HETE
receptor binding in clinical use. Due to the existence
of several isoforms of 12-LO, blockage of the 12-HETE

receptor is a more specific and direct way to correct a disease state in which there is increased production of 12(S)-HETE or the receptors are up-regulated.   This invention therefore, could provide the basis for the

5   development of interventions to reduce cardiovascular disease, diabetes, and cancer.

SUMMARY OF THE INVENTION

The present invention relates to a method of inhibiting the effects of the LO product 12(S)-HETE by

10   blocking 12(S)-HETE receptors comprising the administration of an effective amount of a 12(S)-HETE receptor antagonist or an antibody directed against a cell surface 12(S)-HETE receptor. The blockade of 12(S)-HETE receptors provides a means for ameliorating

15   the proliferative and mitogenic effects of glucose, PDGF or AII-induced 12(S)-HETE production, or direct effects of 12(S)-HETE inflammatory actions.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the binding of tritiated 12(S)-

20   HETE and DuP654 to CHO-AT$_{1a}$ cells at increasing concentrations of unlabeled 12(S).HETE.

Figure 2 shows a competitive binding curve of tritiated and unlabeled 12(S)-HETE with CHO-AT$_{1a}$ cells. Figure 3 shows the effect of the 12(S)-HETE receptor antagonist, DuP654, on AII- and 12(S)-HETE-induced

5　growth in CHO-AT$_{1a}$ cells.

Figure 4 shows the effect of three agents which bind to CHO-AT$_{1a}$ cells, DuP654(a 12(s)HETE receptor antagonist), Losartan (a specific AII $_{1a}$ receptor antagonist) and pertussis toxin, relative to 12(S)-HETE

10　effects.　Inhibition of labeled 12(S)-HETE binding sites on AT$_{1a}$Σ and At$_{1a}$27 (2 cloned overexpressing AT$_{1a}$) cells are shown and pSV neo mock transfected cells.

Figure 5 shows the effect of Losartan on AII and 12(S)-HETE-induced mitogenesis in CHO-AT$_{1a}$ cells.

15　Figure 6 shows the mitogenic effects of AII and 12(S)-HETE on Psv neo mock transfected cells, AT$_{1a}$ expressing cells and AT$_{1b}$ expressing cells.

Figure 7 shows the time course of PAK activation by 12(S)-HETE (10^{-7} M) in CHO-AT$_{1a}$ cells.

20　Figure 8 shows the inhibitory effect of transient transfection of CHO-AT$_{1a}$ cells by a PBD plasmid on 12(S)-HETE-induced PAK activation.

Figure 9 is a representative autoradiogram of phosphorylated MBP bands demonstrating inhibition of

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12(S)-HETE induced PAK activity by the PI 3-kinase

inhibitor, LY294002.

DETAILED DESCRIPTION OF THE INVENTION

Angiotensin II (AII) has been shown to stimulate,

5   through the AII AT, receptor, 12-LO activity in murine

macrophages, Scheidegger et al., J. Biol. Chem.

272:21609-21615 (1997), and in smooth muscle cells,

Natarajan et al., Proc. Natl. Acad. Sci., USA 90:4947-

4951 (1993); Kim et al., Atherosclerol. Thromb. Vasc.

10   Biol. 15:942-948 (1995). Stimulation of the 12-LO

pathway in murine macrophages resulted in an increase

of monocyte chemotaxis (Scheidegger et al., (in press,

1997)), presumably through modification of LDL. This

activity links AII activation of 12-LO to

15   atherosclerotic disease.

The potential mechanisms of AII-induced mitogenic

effects in a Chinese hamster ovary fibroblast cell line

overexpressing the rat vascular type la AII (AT_{1a})

receptor have recently been examined. See Wen et al.,

20   Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220

(1996). AII had mitogenic effects in these cells,

leading to a sustained increase in DNA synthesis as

well as cell number. It was also observed in these

cells that the 12-lipoxygenase product, 12(S)-HETE,
also had direct mitogenic effects in these cells. See
Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40):
C1212-C1220 (1996). Furthermore, 12(S)-HETE did not

5   have any mitogenic effects in mock transfected cells.
The addition of 12(S)-HETE to these CHO-AT$_{1a}$ cells led
to a significant increase in the activity of the key
growth-related kinases, mitogen activated protein
kinase (Wen et al., Am. J. Physiol. 270 (Cell Physiol.

10   40): C1212-C1220 (1996)), and c-jun amino terminal
kinase (Wen et al., Circ. Res. 81:651-655 (1997)).
This work has suggested that over expression of the AT$_{1a}$
receptor plays a role in inducing a putative 12(S)-HETE
receptor, which is supported by the observation that

15   the mitogenic effects of 12(S)-HETE were completely
abrogated by pretreatment of the cells with pertussis
toxin. Thus, the effects of 12(S)-HETE may be mediated
by a Gi protein-coupled receptor. See example 3,
below. Application of AII to CHO-AT$_{1a}$ cells resulted in

20   a 2-fold increase in 12(S)-HETE formation and cell
proliferation. These proliferative effects were
inhibited by the 12-LO inhibitor, baicalein.

In accordance with the present invention, a
12-HETE receptor has been discovered and characterized.

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For the first time, a specific high affinity 12(S)-HETE
receptor has been identified. Chinese hamster ovary
(CHO) fibroblasts that stably overexpress the rat
vascular angiotensin type 1a receptor (CHO-AT$_{1a}$) have

5   been found to carry this receptor. This receptor is
not present in mock transfected cells. Experiments
have been performed which indicate that this receptor
has characteristics of a G-protein coupled receptor.
Furthermore, there is evidence of crosstalk between

10   this receptor and the AT$_{1b}$ receptor, since a specific
antagonist, Losartan, was able to partially block the
binding of 12(S)-HETE to the cells and also blocked the
mitogenic effects of 12(S)-HETE. Furthermore, a 12(S)-
HETE receptor antagonist blocked 12(S)-HETE mitogenic

15   effects and partially blocked AII mitogenic effects.
Increased actions of vasoactive and growth promoting
agents, such as angiotensin II, under pathologic
conditions may up-regulate 12(S)-HETE receptors.
Hence, further studies of this receptor in vascular and

20   other cells, as well as the development of specific
receptor antagonists, are expected to be
therapeutically important.

It has also been found that hyperglycemic
conditions result in both increased monocyte binding to

human aortic endothelial cells (HAEC) and increased

12(S)-HETE and 15-HETE activity. Neutrophil binding is

not increased. In HAEC incubated in vitro with 12-LO

products, increased monocyte binding, JNK activation,

5   and induction of CS-1 fibronectin were detected,

suggesting that the upregulation of 12-LO activity seen

in hyperglycemia may exacerbate atherosclerosis by

stimulating adhesion of monocytes through JNK

activation and CS-1 production. For example, monocytes

10   inabated with 10^{-7} M 12(S)-HETE for 12 minutes at room

temperature or at 37°C prior to monocyte adhesion assay

demonstrated increased adhesion over untreated cells.

See Example 10.

Blockade of 12(S)-HETE receptor binding therefore

15   is a new method of treating disorders associated with

increased 12-lipoxygenase expression and activity.

These diseases include atherosclerotic cardiovascular

disease, glucose and diabetes-induced complications,

cytokine-induced inflammatory cellular effects, and

20   tumor cell growth and metastasis.

The kinetics of radioactive [³H]12(S)-HETE binding

to these cells at 4°C have been examined. These studies

have revealed the presence of specific high affinity

binding sites for 12(S)-HETE on these cells.

Specificity was determined by the observation that this

binding of tritiated 12(S)-HETE was displaced by

unlabeled 12(S)-HETE. A one site fit model yielded a

Kd of 38.4 nM. See Example 1. The binding kinetics of

5   [^{3}H]12(S)-HETE have revealed the presence of specific

high affinity 12(S)-HETE binding sites on CHO-AT$_1$ cells,

but not in mock transfected cells; these results

suggest that AII-induced mitogenic effects involve the

production of reactive oxygen species and LO products

10   via activation of G-protein-coupled receptors.

DuP654 could completely inhibit 12(S)-HETE-induced

mitogenic effects. DuP654 significantly reduced cell

growth induced by either AII or 12(S)-HETE at a

concentration of 0.1 μM. Tritiated 12(S)-HETE binding

15   was also blocked by pertussis toxin (Figure 3).

Pertussis toxin has been shown to ablate 12(S)-HETE-

induced mitogenic effects (Wen et al., Am. J. Physiol.

270 (Cell Physiol. 40): C1212-C1220 (1996)),

implicating the involvement of a G$_i$ protein-coupled

20   receptor. Losartan, a specific angiotensin AT$_{1a}$

receptor antagonist now in clinical use for the

treatment of hypertension, partially blocked tritiated

12(S)-HETE binding (Figure 4). Similarly, it partially

blocked 12(S)-HETE-induced mitogenic effects in these

CHO-AT$_{1a}$ cells, while fully inhibiting AII-induced

proliferative effects (Figure 5). 12(S)-HETE had

mitogenic effects only in CHO-AT$_{1a}$ cells, but not in

mock transfected cells (pSVneo), nor in CHO cells

5   overexpressing the angiotensin AT1b receptor (Figure

6).

       This invention involves a method for inhibiting

the effects of 12(S)-HETE by administration of an

effective amount of a 12(S)-HETE receptor antagonist.

10   The method is useful for the treatment or prophylaxis

of conditions in which 12(S)-HETE receptor activation

contributes to adverse effects. For example, the

method of this invention may be employed for the

treatment or prophylaxis of atherosclerotic

15   cardiovascular disease, glucose-induced complications

of diabetes, cytokine-induced inflammatory diseases and

tumor cell growth and metastasis.

       The 12(S)-HETE receptor antagonist may be any

agent that blocks or significantly inhibits binding of

20   12(S)-HETE to its receptor. Such agents include DuP654

(2-phenylmethyl-1-napthol), Losartan (2-N-butyl-4-

chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-

yl)biphenyl-4-yl)methyl]imidazole, potassium salt),

pertussis toxin, 12(S)-HETE analogs, peptides and

peptide analogs having affinity for the binding site on
the 12(S)-HETE receptor (especially antibodies which
can block 12(S)-HETE receptors), antibodies to the
12(S)-HETE receptor, and the like.

5   The determination of appropriate, well-tolerated
dosage forms for administration to humans for use in
the present invention is within the ordinary skill in
the art. Such dosage forms include tablets, capsules,
syrups, suspensions, drops, injectable solutions,
10   lozenges, implants, transdermal patches, and other
dosage forms well known in the art for enteral or
parenteral administration. Based on in vitro
experiments on the effect of 12(S)-HETE blocking drugs
on 12(S)-HETE binding, a dose of between about 0.5 and
15   about 30 mg/kg/day would be effective in blocking
12(S)-HETE receptors in humans in vivo, and preferably
from about 1 to about 10 mg/kg/day.

The present invention is further illustrated by
the following examples, which are not intended to be
20   limiting.

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EXAMPLES

Example 1

Kinetics of [³H]12(S)-HETE binding to CHO-AT$_{1a}$ cells. Figures 1 and 2 are a competition curves which examine the specificity of [³H]12(S)-HETE binding. CHO-AT$_{1a}$ cells were grown to confluence in 24 well tissue culture dishes in HAM's F12 medium containing 10% fetal calf serum. The cells were then rinsed and placed in fresh medium, HAM's F12/HEPES with no other additives (450 ul per well). Serial dilutions of unlabeled 12(S)-HETE or DuP654 were added to the wells. Commercial unlabeled 12(S)-HETE (BioMol Corp.) was dried and reconstituted in ethanol to obtain a stock solution of 5 mM. DuP654 was dissolved in DMSO to get a stock solution of 5 mM. These were then serially diluted and added in a volume of 1 ul to the wells to obtain the final concentrations indicated. Then [³H]12(S)-HETE (10,000 cpm in a volume of 50 ul per well) was added from a stock solution obtained by adding the tracer to the medium. The plates were then incubated at 4°C with continuous shaking for 2 hr. The cells were then washed 2 times with cold PBS and lysed in 0.3N NaOH (200 ul). Radioactivity in the cell lysates was quantitated in scintillation counter.

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Affinities and binding constants were obtained using
Matlab computer software (Mlab, Civilized Software
Inc., Bethesda, MD).

This experiment revealed the presence of specific
5   high affinity binding sites for 12(S)-HETE on these
cells. A one site fit model yielded a Kd of 38.4 nM.
Specificity was determined by the observation that this
binding of [³H]12(S)-HETE was displaced by unlabeled
12(S)-HETE.

10   Example 2

Reduction of cell growth induced by AII or 12(S)-
HETE.

DuP654 significantly reduced cell growth induced
by either AII or 12(S)-HETE at a concentration of 0.1
15   µM. Complete inhibition of 12(S)-HETE induced
mitogenic effects was seen. See Figure 3.

                              Example 3

Blockade of the 12(S)-HETE receptor by a specific
antagonist.

20   Tritrated 12(S)-HETE binding is blocked by
unlabeled 12(S)-HETE. See Figure 4. DuP654, a 12(S)-
HETE receptor antagonist, was shown also to block
12(S)-HETE at a concentration of 0.1 µM in both $AT_{1a}\Sigma$
and $AT_{1a}27$ cell types, two clones of CHO cells which

overexpress the AII_{1a} receptor. See Figure 4. The
cells were grown as described in Example 1.

<u>Example 4</u>

Blockade of 12(S)-HETE binding by pertussis toxin.
5   Cells were grown as described in Example 1. Prior to
addition of drug (12(S)-HETE or DuP654) to the cells,
the cultures were preincubated in HAM's F12 medium +
0.1% BSA for two hours at $37°C$ with or without 100
ng/ml pertussis toxin. Serial dilutions of unlabeled
10   12(S)-HETE or DuP654 were added, followed by $[^3H]12(S)$-
HETE as described for Example 1. After incubation and
washing, radioactivity in the cell lysates was
quantitated. See Figure 4. As discussed above,
pertussis toxin could also ablate 12(S)-HETE-induced
15   mitogenic effects. This implicates the involvement of
a G_i-protein-coupled receptor.

<u>Example 5</u>

Partial blockade of 12(S)-HETE mitogensis by the
specific angiotensin AT_{1a} receptor antagonist, Losartan,
20   in $CHO-AT_{1a}$ cells. (Figure 5)

$CHO-AT_{1a}$ cells were plated in 12-well dishes (about
5-10,000 cells per well) for 24 hr. in growth medium
consisting of HAM's, F12 + 10% FCS. They were then
serum depleted for 72 hours by replacing the medium

with HAM's F12 + 0.1% BSA. This medium was then
freshly replaced along with AII or 12-HETE (0.1 µM
each) prior to addition of drug to the cells. Losartan
was added as a solution in water to the cells 15 min.

5   prior to the addition of AII or 12-HETE. The final
concentration of Losartan was as indicated in Figure 4.
Fresh medium containing the same concentrations of AII
or 12(S)-HETE plus Losartan was replaced every 48
hours. At the end of 8 days, the medium was removed,

10   and 1 ml trypsin was added per well followed by 1 ml
isoton after 3 min. These trypsinized cells were
counted on a Coulter counter. Losartan partially
blocked 12(S)-HETE-induced mitogenic effects and fully
blocked AII-induced proliferative effects. See Figure

15   5). Losartan also partially blocked [^{3}H] 12-HETE binding
(Figure 4).

Example 6

Dependency of 12(S)-HETE mitogenic effects on
expression of the AT_{1a} receptor.

20   The three cell lines, CHO-AT_{1a}, CHO-AT_{1b} and mock
transfected CHO cells were gifts from Dr. Eric Clauser
(Inserm Unit, Paris, France). These cells were plated
in 12 well dishes in HAM's F12 medium + 10% FCS. After
72 hours serum depletion in HAM's F12 + 0.05% FCS, the

cells were treated with AII or 12(S)-HETE (0.1 μM).
Cells counts (after trypsinization) were taken at 48
hour intervals and fresh medium along with AII or
12-HETE added at these 48 hour intervals. 12(S)-HETE

5   had mitogenic effects only in CHO-AT$_{1a}$ cells, but not in
mock transfected cells (pSVneo), nor in CHO cells
overexpressing the angiotensin AT$_{1b}$ receptor. See
Figure 6.

<u>Example 7</u>

10   PAK activation by 12(S)-HETE.

CHO-AT$_{1a}$ cells were gently washed and placed in
depletion medium (HAM's F-12 medium containing 1 mg\ml
BSA and 20 mM HEPES, ph 7.4) for 72 hours prior to use.
After incubation for 30 minutes, the cells were treated

15   with 10^{-7}M 12(S)-HETE or with ethanol. The 12(S)-HETE
treatment was terminated by washing twice with PBS and
adding 300 μl lysis buffer (50 mM HEPES. pH 7.5,
containing 150 mM NaCl, 5 mM MgCl$_2$, 1 mM EGTA, 50 mM
NaF, 10 mM sodium pyrophosphate, 1% NP-40, 2.5%

20   glycerol and 1 mM Na$_3$VO$_4$ containing the protease
inhibitors phenylmethylsulfonyl fluoride, leupeptin,
and aprotonin) followed by sedimentation at 14,000 xg
at 4 °C for ten minutes. Protein determination was
performed by the Bradford method. The top panel shows

a representative autoradiogram of phosphonycated myelin

basic protein (MBP) bands from a gel. PAK activity was

measured as follows. First, 300 μg of lysate protein

was incubated with PAK antibody (1:20) in lysis buffer

5   overnight at 4°C, followed by incubation with 60 μl of

a 50% slurry of protein A beads for 60 minutes. After

washing three times with lysis buffer and twice with

kinase buffer (50mM HEPES, pH 7.4, 10mM $MgCl_2$, 10mM

$MnCl_2$, and 0.2 dithiothreitol) containing 2 μl MBP, 20

10   μM ATP and 5μCi [γ-^{32}P] ATP, the kinase activity was

measured in 60 μl kinase buffer. After incubation for

30 minutes at 30°C, the reaction was stopped with 5x

Laemmli sample buffer and resolved on a 12% SDS-

polyacrylamide gel, followed by autoradiography. The

15   bottom panel shows the densitometric quantitation of

PAK activity stimulated with 10^{-7} M 12(S)-HETE or

ethanol (control) for the time indicated. Each point

is an average (mean ± SE) from at least 3 separate

experiments. Results are expressed as stimulation over

20   control.

Example 8

Inhibition of 12(S)-HETE induced PAK activation by

transient transaction by a PAK binding domain (PDB)

plasmid.

27

The degree of 12(S)-HETE induce PAK activation was compared in CHO-AT$_{1a}$ cells which had been transiently transfected with a PDB plasmid and cells which had not been transfected.

5　　For the PBD-transfected group, CHO-AT$_{1a}$ cells were transiently transfected with 15 µg PBD plasmid. For the non-PBD-transfected group, CHO-AT$_{1a}$ cells were treated with the same transfection reagents as the PBD-transfected group, but lacking plasmid. Plasmids used

10　　were endotoxin-free and prepared by EndoFree plasmid kit (Qiagen Co.) with the standard protocol. The DNA transfection method used was a cationic liposome-mediated transfection with DOSPER transfection reagent (Boehringer Manahein Co.) following the manufacturer's

15　　instructions. Briefly, the cells were plated the day before the transfection experiment at 3x10^6 cells per 100 mm dish. The next day, cells were washed with Opti-MEMd® reduced serum medium (Gibco BRL) and incubated in 5 ml of HAM's F-12 medium containing 1%

20　　FBS. Plasmid mixture (45 µg DUSPER/15 µg) was prepared and added to each dish. After a 5 hour incubation, the transfection medium was replaced and 8 ml fresh depletion medium (described in Example 7) continuing 1% FBS for overnight incubation was added. The cells were

washed twice with depletion medium, incubated in the
same medium for another 32 hours and harvested. Cells
were then treated with 10^{-7}M 12(S)-HETE or ethanol for
10 minutes. The top panel of Figure 8 illustrates a
5   representative autoradiogram of phosphorylated MBP
bands from a gel. The bottom panel illustrates the
densitometric quantification. Each point is an average
(mean $\pm$ SE) of at least 3 separate experiments.
Results are expressed as stimulation over control. The
10   PAK activity was measured as described in Example 7.

Example 9

.Inhibition of PI 3-kinase by LY294002.

Cells were pretreated with different
concentrations of the PI-3 kinase inhibitor, LY294002
15   or DMSO (control) for 30 minutes, then treated with 10^{-7}
M 12(S)-HETE or ethanol (control) for 10 minutes. PAK
activity was measured as described in Example 7.
Figure 9 shows a representative autoradiogram of
phosphorylated MBP bands from 3 similar experiments.

20   ## Example 10

Increased monocyte adhesion to HAEC by treatment
of monocytes with 12(S)-HETE.

Monocytes were incubated with 10^{-9}M 12(S)-HETE for
12 minutes at room temperature (RT) or 37°C or left

untreated at room temperature and assayed for monocytes

adhesion.   Eight fields were counted for each

experiment.   Results are presented in Table 1.

Table 1 effect of 12(S)-HETE on Monocyte Adhesion to

5   Human Aortic Endothelial Cells

Experiment	NT	12(S)-HETE (RT)	12(S)-HETE (37°C)
1	26.4 ± 12.5	39.6 ± 12.5[1]	39.0 ± 9.3[3]
2	28.3 ± 5.0	45.4 ± 7.6[2]	ND[4]

1. $P = 0.016$
10   2. $P = 0.001$
3. $P = 0.01$
4. ND = not done

CLAIMS

1.   A method for inhibiting the effects of
12-HETE comprising administration of an effective
amount of a 12(S)-HETE receptor blocker.

2.   The method of claim 1 wherein the receptors
are on the cell surface.

3.   The method of claim 2 wherein the in the
cells are selected from the group consisting of
monocytes, endothelial cells, pancreatic islet beta
cells, nerve cells, cardiac fibroblasts, cardiac
myocytes and vascular smooth muscle cells.

4.   The method of claim 1 wherein the
administration of the compound is to an animal.

5.   The method of claim 4 wherein the animal is a
mammal.

6.   The method of claim 4 wherein the animal is a
human.

7.   The method of claim 1 wherein the blocking of
the receptor inhibits 12(S)-HETE binding.

8.   The method of claim 1 wherein the blocking of
the receptor inhibits receptor activation.

9.   The method of claim 1 wherein the blocking of
the receptor inhibits cell growth.

10. The method of claim 1 wherein the blocking of the receptor inhibits inflammatory cell damage.

11. The method of claim 1 wherein the blocking of the receptor inhibits cell death.

12. The method of claim 1 wherein the blocking of the receptor reduces monocyte adhesion.

13. The method of claim 1 wherein the blocking of the receptor reduces VEGF producion.

14. The method of claim 1 wherein the blocking of the receptor reduces PAK activation.

15. The method of claim 1 wherein the 12(S)-HETE receptor blocker is a 12(S)-HETE receptor antagonist.

16. The method of claim 1 wherein the 12(S)-HETE receptor blocker is an antibody.

17. A method of suppressing the activation of 12(S)-HETE receptors comprising the administration of a compound which prevents the binding of endogenous receptor agonists to the receptor.

18. A method for the treatment or prophylaxis of a disease in which 12(S)-HETE receptor activation contributes to adverse effects which comprises administering an effective amount of a 12(S)-HETE

5   receptor blocker.

19.   The method of claim 18, wherein the disease
is atherosclerotic cardiovascular disease, glucose-
induced complications of diabetes, Parkinson's disease,
Alzheimer's disease, stroke-induced nerve damage,

5   cytokine induced inflammatory disease or tumor cell
growth or metastasis.

20.   The method of claim 19, wherein the 12-HETE
receptor blocker is selected from the group consisting
of DuP654, Losartan, pertussis toxin, a 12(S)-HETE
analog, an antibody to the 12(S)-HETE receptor, a

5   peptide which binds to the 12(S)-HETE receptor and a
peptide analog which binds to the 12(S)-HETE receptor.

21.   The method of claim 18, wherein the 12-HETE
receptor blocker is administered in a dose of from
about 0.5 to about 300 mg/kg/day.

22.   The method of claim 21, wherein the 12-HETE
receptor blocker is administered in a dose of from
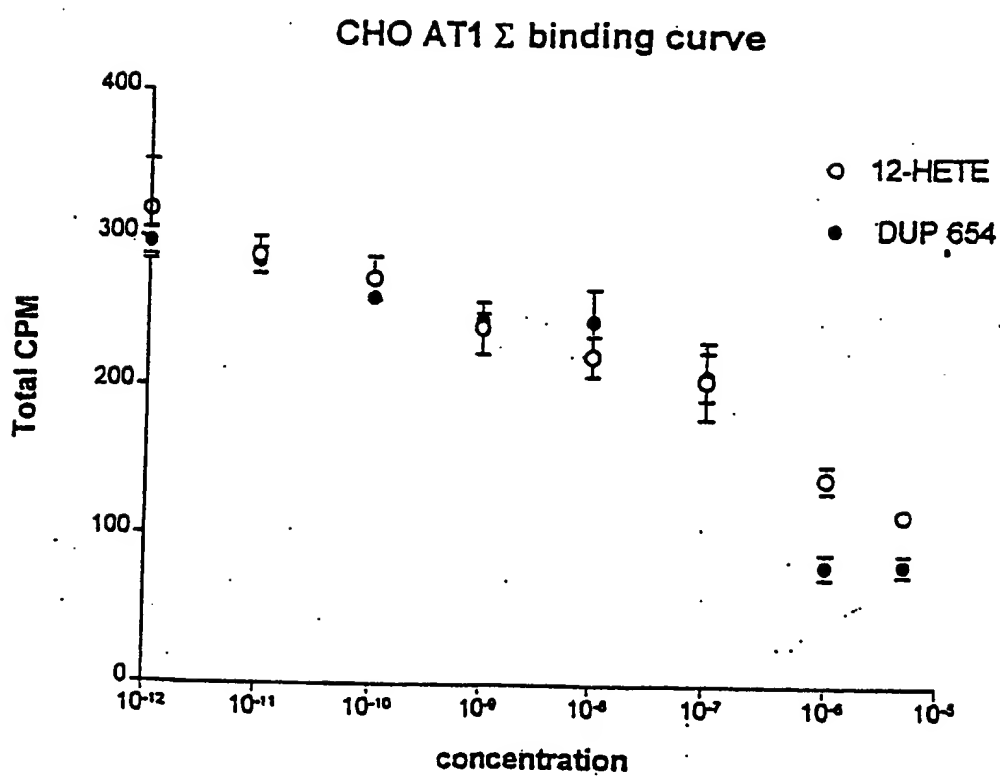about 1 to about 10 mg/kg/day.

FIGURE 1

[³H]12(S)-HETE Binding to CHO-AT$_{1a}$ Cells

CHO AT1 Σ binding curve

o 12-HETE

• DUP 654

FIGURE 2

Competition of Cold 12(S)-HETE for 12(S)-[³H]HETE Binding to CHO-AT$_{1a}$ Cells.
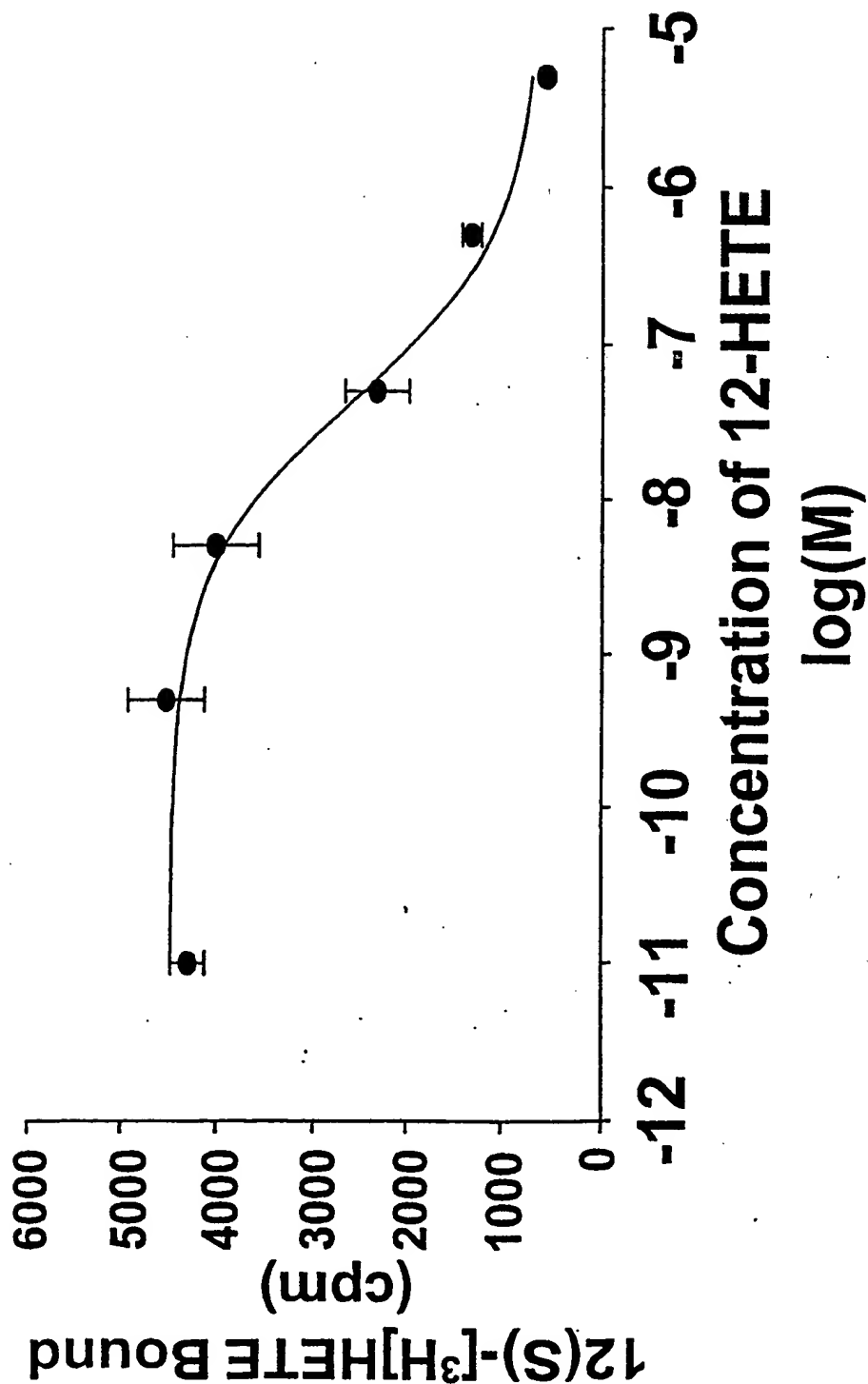
FIGURE 3

Effect of DuP654(12-HETE receptor antagonist) on AII and 12-HETE- induced Growth in CHO-AT$_{1a}$ cells

* p<0.001 vs control
** p<0.01 vs AII
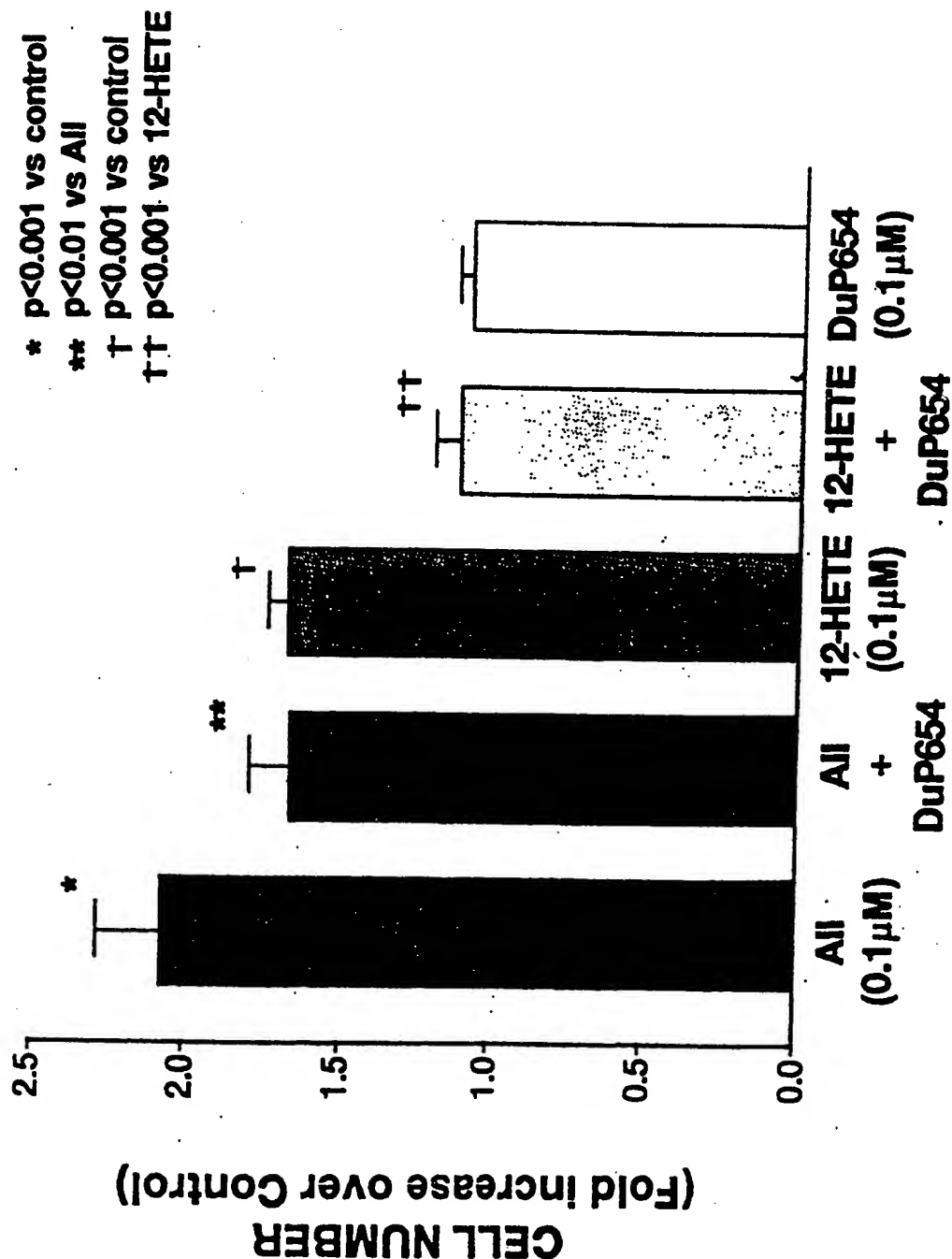† p<0.001 vs control
†† p<0.001 vs 12-HETE

FIGURE 4

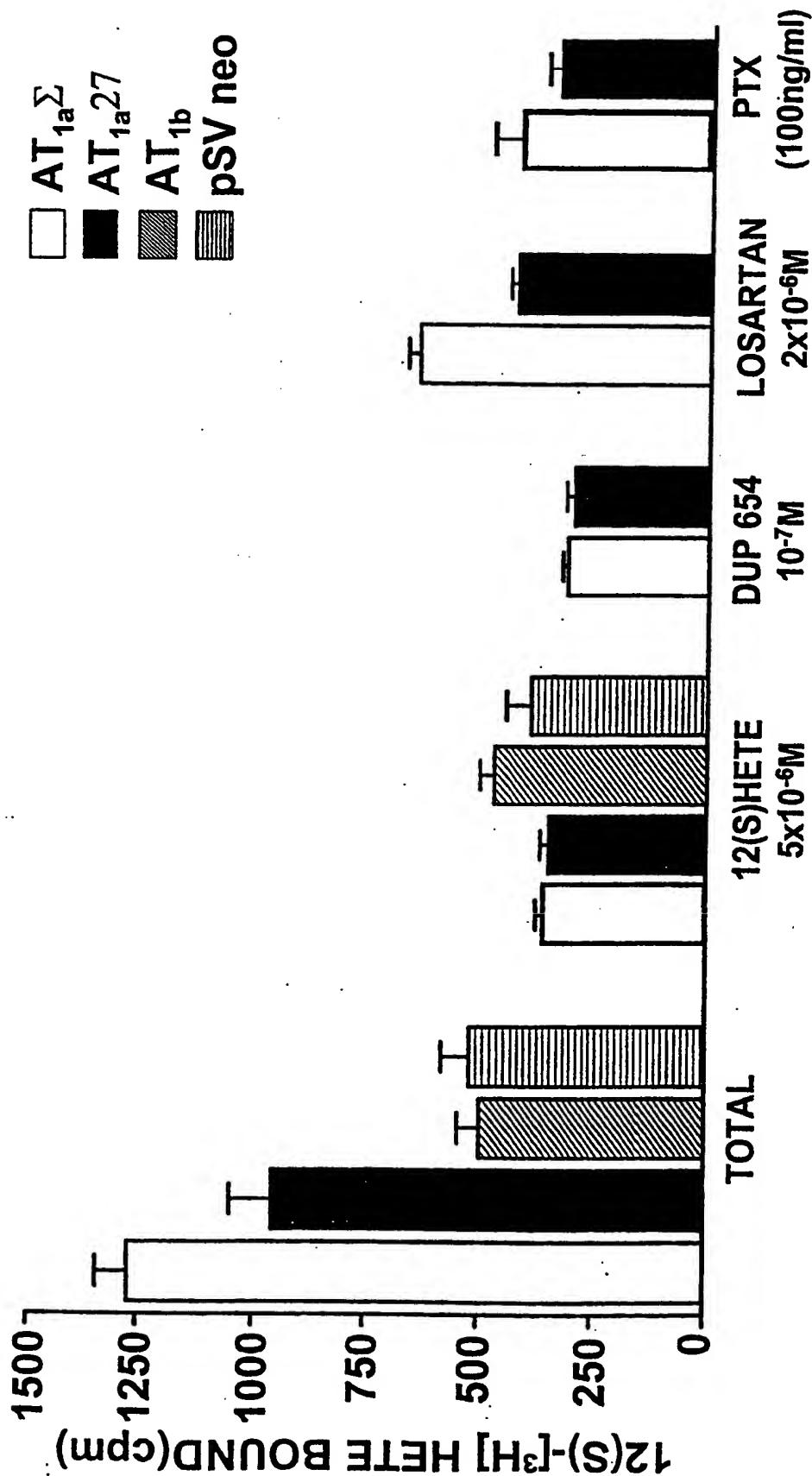# Effect of Agents on 12(S)-[³H] HETE Binding to CHO-AT$_{1a}$ Cells

FIGURE 5

Effect of Losartan(Los) on AII and 12-HETE-induced Mitogenesis in CHO-AT$_{1a}$ cells

* p<0.001 vs control
** p<0.001 vs AII
† p<0.001 vs control
†† p<0.01 vs 12-HETE
††† p<0.001 vs 12-HETE
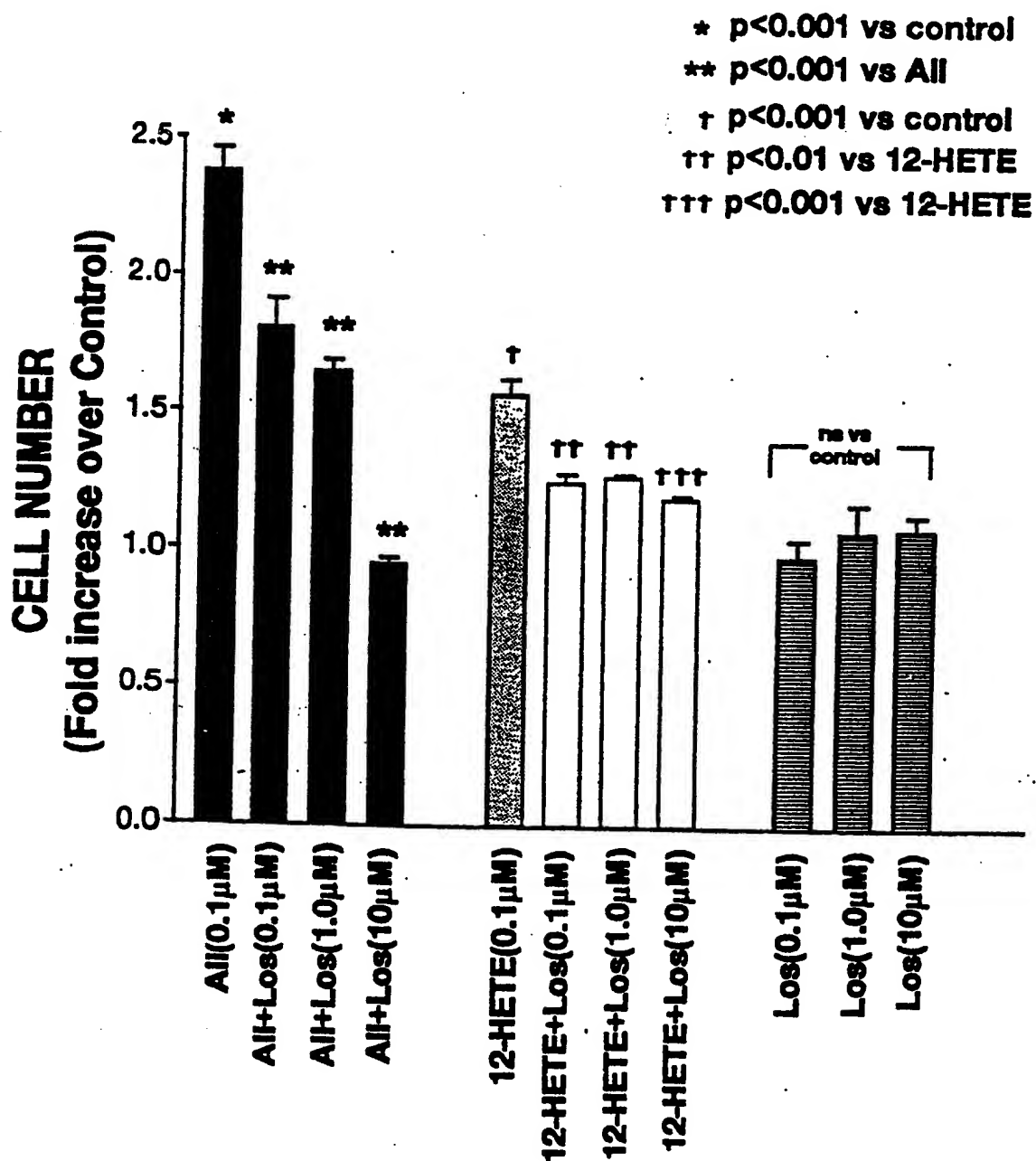
FIGURE 6

CHO
Growth curve
0.05% BSA

○ ctrl

● AII 10^{-7}M

▽ 12HETE 10^{-7}M
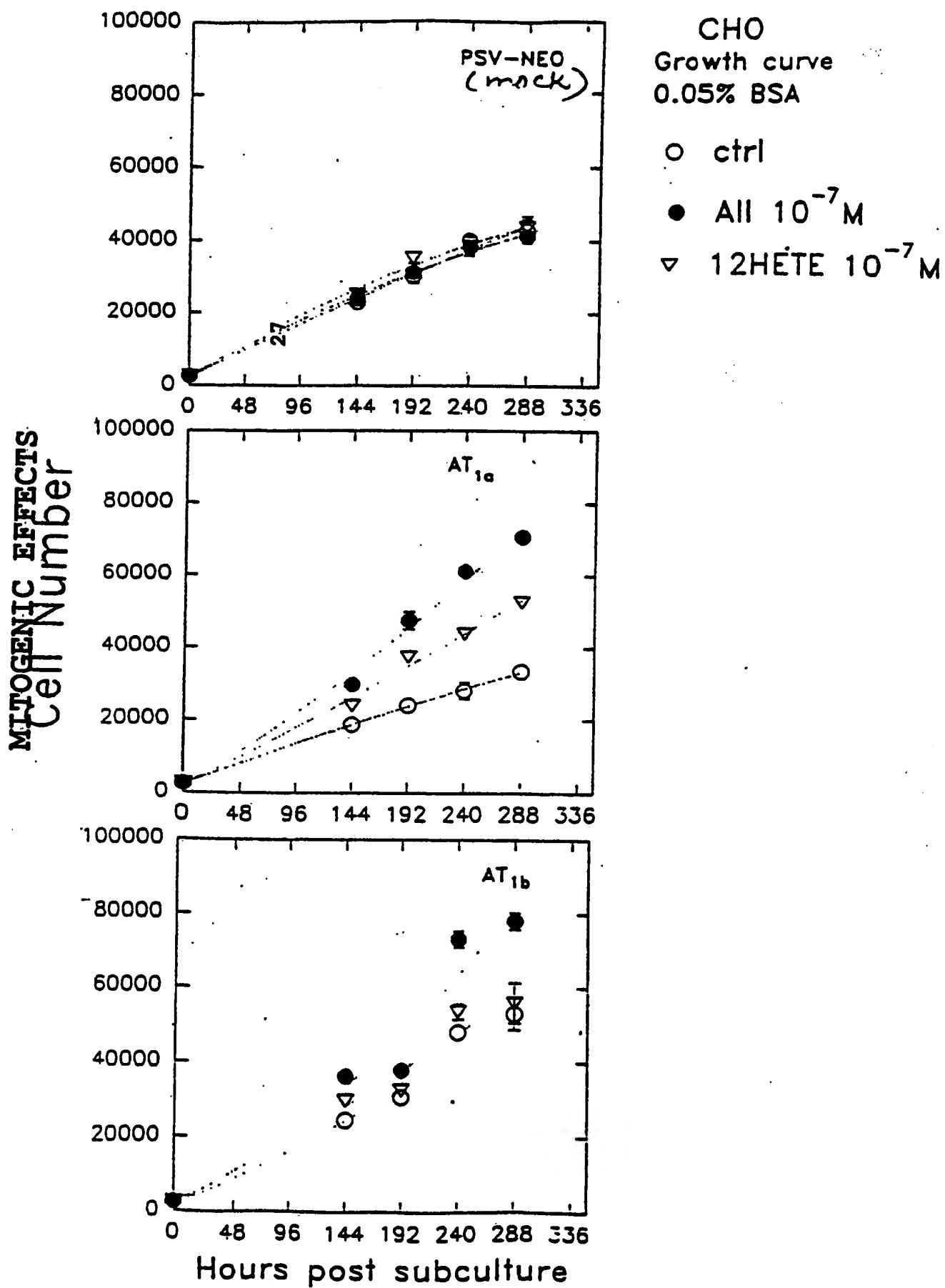
FIGURE 7

FIGURE 8

FIGURE 9

control · 12-HETE · 0.15μM LY · 0.15μM LY+ 12-HETE · 1.5μM LY · 1.5μM LY+ 12-HETE

← MBP

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K31/415   A61K38/16   A61K31/20   A61K39/395   A61K31/05

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 34943 A (HOPE CITY ;NADLER JERRY L (US); NATARAJAN RAMA (US)) 7 November 1996 see page 3, line 28 – page 5, line 29 see page 23, line 9 – page 24, line 29 see page 26, line 32 – page 27, line 27 see page 31, line 24 – line 27 see page 32, line 10 – line 16 see page 43, line 20 – line 22 see page 49, line 33 – line 34 see claims 6,21,25,29-31 -/--	1-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 1999	02/03/1999

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4

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ARENBERGER, PETR ET AL: "The lipoxygenase inhibitor 2-phenylmethyl-1-naphthol (DuP 654) is a 12(S)-hydroxyeicosatetraenoic acid receptor antagonist in the human epidermal cell line SCL-II" SKIN PHARMACOL. (1993), 6(2), 148-51 , XP002093788 cited in the application see the whole document	1,2, 4-10,15, 17-22
Y	---	3,11
Y	BLEICH D ET AL: "The stress-activated c-jun protein kinase (JNK) is stimulated by lipoxygenase pathway product 12-HETE in RIN m5F cells" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATION, vol. 230, 1997, pages 448-451, XP002093789 cited in the application see the whole document ---	3,11
X	WEN Y ET AL: "Mechanisms of ANGII-induced mitogenic responses: role of 12-lipoxygenase and biphasic MAP kinase" AM.J.PHYSIOLOGY:CELL PHYSIOLOGY, vol. 40, no. 4, October 1996, pages c1212-c1220, XP002093790 cited in the application see the whole document ---	1-9,14, 18-22
A	NATARAJAN R ET AL: "MECHANISM OF ANGIOTENSIN II-INDUCED PROLIFERATION IN BOVINE ADRENOCORTICAL CELLS" ENDOCRINOLOGY, vol. 131, no. 3, September 1992, pages 1174-1180, XP000601236 see the whole document -----	1-15,21, 22

4

Box I   Obs rvations wh re certain laims w r f und uns archabl (C ntinuation of item 1 f first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority. namely:

   Remark: Although claim(s) 1-22
   is(are) directed to a method of treatment of the human/animal
   body, the search has been carried out and based on the alleged
   effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out. specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application. as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all
   searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee. this Authority did not invite payment
   of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant. this International Search Report
   covers only those claims for which fees were paid. specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9634943 A	07-11-1996	AU 5637796 A CA 2220156 A EP 0824583 A	21-11-1996 07-11-1996 25-02-1998

PCT

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(54) Title: 12(S)–HETE RECEPTOR BLOCKERS

(57) Abstract

The 12–lipoxygenase product, 12(S)–HETE, mediates hyperproliferative and hyperplastic responses seen in atherosclerosis, diabetes, Parkinson's disease, Alzheimer's, stroke–induced nerve damage and cancer. 12–HETE also mediates inflammation and cell death in some cell systems, particularly B–islet cells of the pancreas. The present invention involves amelioration of disease states mediated by 12(S)–HETE by blocking specific 12(S)–HETE receptors.

12(S)-HETE RECEPTOR BLOCKERS

This application claims priority from provisional application 60/062,335, filed October 15, 1997.

STATEMENT REGARDING FEDERALLY SUPPORTED RESEARCH

5   This invention was made with government support under Grant No. DK 39721 awarded by the National Institutes of Health (NIDDK). The government may have certain rights in the invention.

BACKGROUND OF THE INVENTION

10 **Field of the Invention**

The present invention relates to blockade of the 12(S)-HETE cell surface receptor as a treatment for conditions of the body which result from stimulation or overstimulation of the receptor. 12(S)-HETE, a product

15 of the 12-lipoxygenase pathway, mediates the hyperproliferative and inflammatory responses present in such diseases as atherosclerosis, psoriasis, diabetes, and cancer. 12(S)-HETE also mediates inflammatory responses and cell death in some cell

20 types, particularly pancreatic islet beta cells and nerve cells. Blockade of the 12(S)-HETE receptor

ameliorates the symptoms and arrests the mitogenic

cellular responses.

Background

Lipoxygenases (LO) are metabolic enzymes which

5   catalyze the stereospecific oxygenation of

polyunsaturated fatty acids to hydroperoxy fatty acids

(Hamberg et al., J. Biol. Chem. 242:5329-5335 (1967)).

The physiological function of 12-LO, the mammalian

enzyme which catalyzes the oxygenation of arachidonic

10   acid to (S)-12-hydroperoxyeicosatetraenoic acid (12-

HPETE) and (S)-12-hydroxyeicosatetraenoic acid (12(S)-

HETE), is unclear.

12-LO exists as two isoforms which are the

products of different genes, leukocyte-type 12-LO and

15   platelet-type 12-LO, which share 65% homology at the

amino acid level (Izumi et al., Proc. Natl. Acad. Sci.,

USA 87:7477-7481 (1990); Funk et al., Proc. Natl. Acad.

Sci. USA 87:5638-5642 (1990)). The products of the 12-

LO pathway, such as 12(S)-HETE, have been shown to play

20   important roles in diseases such as atherosclerosis,

diabetes, and cancer.

12(S)-HETE has direct mitogenic and hypertrophic

effects in vascular cells. It is also a potent

chemoattractant for vascular smooth muscle cells (VSMC) and can activate oncogenes such as c-fos and ras and key growth-related kinases such as mitogen-activated protein kinases (ERK, JNK, PAK, p38) and protein

5   kinase C. New results also indicate that 12(S)-HETE can directly increase monocyte binding. Human aortic endothelial cells incubated with 12(S)-HETE for four hours prior to monocyte adhesion assays resulted in an average increase of 3-fold (range of 1.5-5 fold) in

10   monocyte binding as compared to untreated cells. In addition, glucose-induced monocyte adhesion was abrogated by the inhibition of 12-LO using both phenidone, a non-specific LO inhibitor, and baicalein, a more specific 12-LO inhibitor. The adhesion caused

15   by 12-LO products appears to be monocyte-specific.

The 12-LO pathway is activated in pancreatic islets by cytokines and may participate in islet cell destruction. In inflammatory diseases, this pathway plays crucial roles in transmitting distinctive signals

20   within the cell. Using inhibitors of the 12-LO enzyme pathway, researchers have been able to prevent inflammation and cellular damage. Furthermore, VSMC cultured under high glucose (HG) conditions produce increased amounts of 12(S)-HETE (Natarajan et al.,

4

Proc. Natl. Acad. Sci. USA 90:4947-4951 (1993). Thus,
this pathway may be key to the accelerated
cardiovascular disease observed in diabetes.

The LO pathway also plays a role in the growth-
5   promoting effects of angiotensin II (AII) and in the
chemotactic effects of platelet-derived growth factor:
the products of the 12-LO pathway, and 12(S)-HETE in
particular, are associated with the hypertrophic,
hyperplastic, and mitogenic effects induced by AII.
10   Wen et al., 271 Am. J. Physiol. (40 Cell Physiol.)
C1212-C1220 (1996); (Natarajan et al., Hypertension
23:I142-I147 (1994)). The proliferative effects of AII
are inhibited by baicalein, a LO inhibitor. The
mitogenic effects of 12(S)-HETE are similar to those of
15   AII and are abrogated by pertussis toxin, implicating a
G-protein mechanism. The 12-LO enzyme pathway is known
to generate proinflammatory mediators in a variety of
cells (O.R. Etingin et al., J. Lipid Res. 31:299-305
(1990); V.A. Folcik and M.K. Cathcart J. Lipid Res.
20   34:69-79 (1993)). Human and rat pancreatic B-cells
specifically express active leukocyte type 12-LO (V.P.
Shannon et al., Am. J. Physiol. 263:E828-E836 (1992):
D.S. Bleich et al., Endocrinol. 136:5736-5744 (1995)).
Recent evidence implicates products of the 12-LO

5

pathway in nerve cell death associated with Parkinson's

disease, Alzheimer's disease and other inflammatory

nerve cell conditions (Neuron 19:453-463 (1997)).

Because 12(S)-HETE has several biological effects

5   linked to cellular growth in vascular smooth muscle and

cardiac fibroblasts (Natarajan et al., Hypertension

23:I142-I147 (1994); Wen et al., Am. J. Physiol.

211:C1212-C1220 (1996)), it is implicated in the

etiology of cardiovascular disease. Further evidence

10   that 12(S)-HETE is responsible for the cellular

responses seen in cardiovascular disease in diabetic

patients includes the fact that monocyte binding to

cultured human aortic endothelial cells increases in

chronic high glucose conditions, and that this is

15   coincident with increased formation of LO products such

as 12(S)-HETE. (Kim et al., Diabetes 43:1103-1107

(1994)). Furthermore, treatment of aortic endothelial

cells with 12(S)-HETE increases monocyte binding,

likely by stimulating JNK activity and inducing CS-1.

20   12(S)-HETE can also stimulate vascular endothelial

growth factor (VEGF) gene expression in vascular smooth

muscle (Am. J. Physiol. 273: H2224-H2231 (1997)). VEGF

has been linked to angiogenesis in diabetic

retinopathy, tumor growth and atherosclerotic vascular
disease.

12(S)-HETE is also regarded as a mediator of
inflammation and hyperproliferation of the skin
(Arenberger et al., Skin Pharmacol. 6:148-151 (1993);
Gross et al., J. Invest. Dermatol. 94:446-451 (1990))
and is therefore implicated in skin diseases.
12(S)-HETE has been shown to enhance tumor cell
adhesion to endothelial cells. (Honn et al., Cancer
Metastasis Rev. 13:365-396 (1994)). 12(S)-HETE can
directly increase p21 activated kinase (PAK). The
effect appears to be through activation of small GTP
binding proteins such as RAC and through activation of
PI3K.

The precise mechanisms of 12(S)-HETE action are
not clear, however recent studies have shown that the
LO product, 12(S)-HETE, activates c-jun amino terminal
kinase (JNK) (Wen et al., Circ. Res. 81:651-655
(1997)). JNK is a small GTP-binding protein and a
member of the MAP kinase family which is involved in
cellular growth, inflammation, and apoptosis (Force et
al., Circ. Res. 78:947-953 (1994)) and in cell cycle
progression through G_1 (Olson et al., Science 269:1270-
1272 (1995)). Evidence shows that JNK can serve as a

positive or negative modulator of cell growth in different cells. Olson et al., 269 Science 1270-1272 (1995); Yan et al., 372 Nature 798-800 (1994). 12(S)-HETE activation of JNK may also be the mediator of cytokine-induced pancreatic B-cell damage (Bleich et al., Biochem. Biophys. Res. Commun. 230:448-451 (1997)).

Newer evidence indicates that the growth factor and potent vasoconstrictor AII, linked to type-1 receptor activation, can activate JNK and PAK (Wen et al., Circ. Res. 81:651-655 (1997); Schmitz et al., Circ. Res. 82:1272-1278 (1998)). Furthermore, AII can modulate serum deprivation-induced apoptosis by increasing JNK activity in vascular smooth muscle cells, Sueror et al., Circulation, Supp. 1, I-281 (1994), mediated by lipids derived from the 12-LO pathway, such as 12(S)-HETE. This indicates that 12-LO products participate in JNK activation at least in part through G_1-protein signaling. The ability of pertussis toxin to block the activation of JNK by 12(S)-HETE also supports the theory that 12(S)-HETE is a mediator of AII-induced JNK activation through a G_1-mediated pathway.

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While several studies have demonstrated the potent biological effects of lipoxygenase products, the mechanisms of action of these effects are not known. Some reports have hinted at the presence of 12(S)-HETE

5   receptors on transformed cells. Binding sites for 12(S)-HETE have been detected in carcinoma cells (Herbertsson and Hammarstrom, FEBS 298:249-252 (1992), on melanoma cells (Liu et al., Proc. Natl. Acad. Sci. USA 92:9323-9327 (1995), and in a human epidermal cell

10   line (Gross et al., J. Invest. Dermatol. 94:446-451 (1990); Suss et al., Exptl. Cell Res. 191(2):204-208 (1990)).

The 12(S)-HETE receptors described in carcinoma cells are cytosolic receptors (Herbertsson and

15   Hammarstrom, Biochem. Biophys. Acta 1244:191-197 (1995)), activation of which may mediate 12(S)-HETE induced mRNA production of genes coding for the integrin $\alpha_{IIb}\beta_3$ (Chang et al., Biochem. Biophys. Res. Comm. 176:108-113 (1991)). The localization of this

20   receptor is different from plasma cell membrane receptors coupled to a G-protein and acting through second messengers.

12(S)-HETE receptors on the cell surface of murine melanoma cells have been described. These receptors

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stimulate the second messengers diacylglycerol and
inositol phosphate, via a G-protein mechanism, resulting
in protein kinase C_2 activation. (Liu et al., Proc.
Natl. Acad. Sci. USA 92:9323-9327 (1995)). The binding
5   of 12(S)-HETE to these receptors was blocked by 13(s)-
hydroxyoctadecadienoic acid, a LO metabolite of
linoleic acid, ablating the 12(S)-HETE increased
adhesion of the cells to fibronectin. These authors
suggest 12(S)-HETE may act in a "cytokine" fashion to
10   regulate responses of adjacent tumor cells, endothelial
cells, and platelets.

Receptors for 15-HETE have been identified in
mast/basophil (PT-18) cells and were shown to possess
properties of G-protein-coupled receptors (Vonakis and
15   Vanderhoek, J. Biol. Chem. 267:23625-23631 (1992).
Specific binding of 15-HETE to these receptors
stimulated 5-LO, and while 12(S)-HETE was found to be
an effective competitor of [³H]15-HETE binding to PT-18
cells, suggesting that 12(S)-HETE binds to the specific
20   15-HETE receptor, the binding of 12(S)-HETE did not
stimulate the lipoxygenase. Very recent studies have
indicated the activation of a cell surface G-protein-
coupled 5-HETE receptor in neutrophils (Capadici
et al., J. Clin. Invest. 102:165-175 (1998)).

10

The high affinity 12(S)-HETE-specific receptors in
a human epidermal carcinoma cell line were induced by
γ-IFN (Gross et al., J. Invest. Dermatol. 94:446-451
(1990)). Saturation binding of 12(S)-HETE to these

5   receptors did not stimulate cell growth, therefore, the
function of these receptors in the skin is entirely
speculative, and not related to the AII-induced
cellular effects mediated by cell surface 12(S)-HETE
receptors in fibroblasts overexpressing the AII

10   receptor and potentially in vascular smooth muscle
cells. Two recent studies have indicated two
additional agents which could reduce 12(S)-HETE binding
(Kemeny and Ruzicka, Agents Actions 32:339-342 (1991);
Kemeny et al., Arch. Dermatol. Res. 283:333-336

15   (1991)).

Specific inhibitors of 12-LO have been described.
Gorins et al., J. Med. Chem. 39:4871-4878 (1996). In
that study, a series of substituted
(carboxyalkyl)benzyl ethers were found to be selective

20   inhibitors of leukocyte-type 12-LO. These inhibitors
of 12-LO acted by serving as structural analogs for the
enzyme. Gorins et al., J. Med. Chem. 39:4871-4878
(1996). The 5-LO inhibitor, 2-phenylmethyl-1-naphthol
(DuP654), has also been shown to specifically inhibit

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binding of 12(S)-HETE to receptors on the human

epidermal cell line SCL-II. Arenberger et al., Skin

Pharmacol 6:148-151 1993).

In vivo, inhibition of 12-LO has lowered blood

5   pressure in several models of hypertensive animals,

including rats (Stern et al., Am. J. Physiol. 257:H434-

H443 (1989); Nozawa et al., Am. J. Physiol. 259:H1447-

H1780 (1990)). In addition, blockage of 12-LO activity

has alleviated the growth-factor induced effects of

10 12-HETE in vascular cells. This, along with the known

increased expression of 12-LO observed in animal models

of diabetes (Gu et al., Am. Diabet. Assoc. Meeting

(1996); Natarajan et al., Intl. Aldosterone Meeting

(1998)) and diabetes induced accelerated

15 atherosclerosis (Gerrity et al., Circulation I175

(1997)) strongly implicate 12-HETE and the 12-LO

pathway in the etiology of these diseases. The harmful

effects of 12-LO activation are ameliorated by blocking

the production of 12(S)-HETE, providing the rationale

20 for a method of treatment which focusses on preventing

12(S)-HETE binding to its receptor.

There is currently no inhibitor of 12(S)-HETE

receptor binding in clinical use. Due to the existence

of several isoforms of 12-LO, blockage of the 12-HETE

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receptor is a more specific and direct way to correct a
disease state in which there is increased production of
12(S)-HETE or the receptors are up-regulated.   This
invention therefore, could provide the basis for the
5   development of interventions to reduce cardiovascular
disease, diabetes, and cancer.

SUMMARY OF THE INVENTION

The present invention relates to a method of
inhibiting the effects of the LO product 12(S)-HETE by
10   blocking 12(S)-HETE receptors comprising the
administration of an effective amount of a 12(S)-HETE
receptor antagonist or an antibody directed against a
cell surface 12(S)-HETE receptor.   The blockade of
12(S)-HETE receptors provides a means for ameliorating
15   the proliferative and mitogenic effects of glucose,
PDGF or AII-induced 12(S)-HETE production, or direct
effects of 12(S)-HETE inflammatory actions.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the binding of tritiated 12(S)-
20   HETE and DuP654 to CHO-AT$_{1a}$ cells at increasing
concentrations of unlabeled 12(S).HETE.

Figure 2 shows a competitive binding curve of
tritiated and unlabeled 12(S)-HETE with CHO-AT$_{1a}$ cells.
Figure 3 shows the effect of the 12(S)-HETE receptor
antagonist, DuP654, on AII- and 12(S)-HETE-induced

5   growth in CHO-AT$_{1a}$ cells.

Figure 4 shows the effect of three agents which
bind to CHO-AT$_{1a}$ cells, DuP654(a 12(s)HETE receptor
antagonist), Losartan (a specific AII $_{1a}$ receptor
antagonist) and pertussis toxin, relative to 12(S)-HETE

10   effects. Inhibition of labeled 12(S)-HETE binding
sites on AT$_{1a}\Sigma$ and At$_{1a}$27 (2 cloned overexpressing AT$_{1a}$)
cells are shown and pSV neo mock transfected cells.

Figure 5 shows the effect of Losartan on AII and
12(S)-HETE-induced mitogenesis in CHO-AT$_{1a}$ cells.

15   Figure 6 shows the mitogenic effects of AII and
12(S)-HETE on Psv neo mock transfected cells, AT$_{1a}$
expressing cells and AT$_{1b}$ expressing cells.

Figure 7 shows the time course of PAK activation
by 12(S)-HETE (10^{-7} M) in CHO-AT$_{1a}$ cells.

20   Figure 8 shows the inhibitory effect of transient
transfection of CHO-AT$_{1a}$ cells by a PBD plasmid on
12(S)-HETE-induced PAK activation.

Figure 9 is a representative autoradiogram of
phosphorylated MBP bands demonstrating inhibition of

12(S)-HETE induced PAK activity by the PI 3-kinase

inhibitor, LY294002.

DETAILED DESCRIPTION OF THE INVENTION

Angiotensin II (AII) has been shown to stimulate,

5   through the AII AT, receptor, 12-LO activity in murine

macrophages, Scheidegger et al., J. Biol. Chem.

272:21609-21615 (1997), and in smooth muscle cells,

Natarajan et al., Proc. Natl. Acad. Sci., USA 90:4947-

4951 (1993); Kim et al., Atherosclerol. Thromb. Vasc.

10   Biol. 15:942-948 (1995). Stimulation of the 12-LO

pathway in murine macrophages resulted in an increase

of monocyte chemotaxis (Scheidegger et al., (in press,

1997)), presumably through modification of LDL. This

activity links AII activation of 12-LO to

15   atherosclerotic disease.

The potential mechanisms of AII-induced mitogenic

effects in a Chinese hamster ovary fibroblast cell line

overexpressing the rat vascular type 1a AII (AT_{1a})

receptor have recently been examined. See Wen et al.,

20   Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220

(1996). AII had mitogenic effects in these cells,

leading to a sustained increase in DNA synthesis as

well as cell number. It was also observed in these

cells that the 12-lipoxygenase product, 12(S)-HETE,
also had direct mitogenic effects in these cells. See
Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40):
C1212-C1220 (1996). Furthermore, 12(S)-HETE did not

5   have any mitogenic effects in mock transfected cells.
The addition of 12(S)-HETE to these CHO-AT$_{1a}$ cells led
to a significant increase in the activity of the key
growth-related kinases, mitogen activated protein
kinase (Wen et al., Am. J. Physiol. 270 (Cell Physiol.

10 40): C1212-C1220 (1996)), and c-jun amino terminal
kinase (Wen et al., Circ. Res. 81:651-655 (1997)).
This work has suggested that over expression of the AT$_{1a}$
receptor plays a role in inducing a putative 12(S)-HETE
receptor, which is supported by the observation that

15 the mitogenic effects of 12(S)-HETE were completely
abrogated by pretreatment of the cells with pertussis
toxin. Thus, the effects of 12(S)-HETE may be mediated
by a Gi protein-coupled receptor. See example 3,
below. Application of AII to CHO-AT$_{1a}$ cells resulted in

20 a 2-fold increase in 12(S)-HETE formation and cell
proliferation. These proliferative effects were
inhibited by the 12-LO inhibitor, baicalein.

In accordance with the present invention, a
12-HETE receptor has been discovered and characterized.

For the first time, a specific high affinity 12(S)-HETE receptor has been identified. Chinese hamster ovary (CHO) fibroblasts that stably overexpress the rat vascular angiotensin type 1a receptor (CHO-AT$_{1a}$) have

5   been found to carry this receptor. This receptor is not present in mock transfected cells. Experiments have been performed which indicate that this receptor has characteristics of a G-protein coupled receptor. Furthermore, there is evidence of crosstalk between

10   this receptor and the AT$_{1b}$ receptor, since a specific antagonist, Losartan, was able to partially block the binding of 12(S)-HETE to the cells and also blocked the mitogenic effects of 12(S)-HETE. Furthermore, a 12(S)-HETE receptor antagonist blocked 12(S)-HETE mitogenic

15   effects and partially blocked AII mitogenic effects. Increased actions of vasoactive and growth promoting agents, such as angiotensin II, under pathologic conditions may up-regulate 12(S)-HETE receptors. Hence, further studies of this receptor in vascular and

20   other cells, as well as the development of specific receptor antagonists, are expected to be therapeutically important.

It has also been found that hyperglycemic conditions result in both increased monocyte binding to

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human aortic endothelial cells (HAEC) and increased
12(S)-HETE and 15-HETE activity. Neutrophil binding is
not increased. In HAEC incubated _in vitro_ with 12-LO
products, increased monocyte binding, JNK activation,

5   and induction of CS-1 fibronectin were detected,
suggesting that the upregulation of 12-LO activity seen
in hyperglycemia may exacerbate atherosclerosis by
stimulating adhesion of monocytes through JNK
activation and CS-1 production. For example, monocytes

10   inabated with 10^{-7} M 12(S)-HETE for 12 minutes at room
temperature or at 37°C prior to monocyte adhesion assay
demonstrated increased adhesion over untreated cells.
See Example 10.

Blockade of 12(S)-HETE receptor binding therefore

15   is a new method of treating disorders associated with
increased 12-lipoxygenase expression and activity.
These diseases include atherosclerotic cardiovascular
disease, glucose and diabetes-induced complications,
cytokine-induced inflammatory cellular effects, and

20   tumor cell growth and metastasis.

The kinetics of radioactive [3H]12(S)-HETE binding
to these cells at 4°C have been examined. These studies
have revealed the presence of specific high affinity
binding sites for 12(S)-HETE on these cells.

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Specificity was determined by the observation that this
binding of tritiated 12(S)-HETE was displaced by
unlabeled 12(S)-HETE. A one site fit model yielded a
Kd of 38.4 nM. See Example 1. The binding kinetics of
5   [^{3}H]12(S)-HETE have revealed the presence of specific
high affinity 12(S)-HETE binding sites on CHO-AT$_1$ cells,
but not in mock transfected cells; these results
suggest that AII-induced mitogenic effects involve the
production of reactive oxygen species and LO products
10   via activation of G-protein-coupled receptors.

DuP654 could completely inhibit 12(S)-HETE-induced
mitogenic effects. DuP654 significantly reduced cell
growth induced by either AII or 12(S)-HETE at a
concentration of 0.1 μM. Tritiated 12(S)-HETE binding
15   was also blocked by pertussis toxin (Figure 3).
Pertussis toxin has been shown to ablate 12(S)-HETE-
induced mitogenic effects (Wen et al., Am. J. Physiol.
270 (Cell Physiol. 40): C1212-C1220 (1996)),
implicating the involvement of a G$_i$ protein-coupled
20   receptor. Losartan, a specific angiotensin AT$_{1a}$
receptor antagonist now in clinical use for the
treatment of hypertension, partially blocked tritiated
12(S)-HETE binding (Figure 4). Similarly, it partially
blocked 12(S)-HETE-induced mitogenic effects in these

CHO-AT$_{1a}$ cells, while fully inhibiting AII-induced

proliferative effects (Figure 5). 12(S)-HETE had

mitogenic effects only in CHO-AT$_{1a}$ cells, but not in

mock transfected cells (pSVneo), nor in CHO cells

5   overexpressing the angiotensin AT1b receptor (Figure

6).

This invention involves a method for inhibiting

the effects of 12(S)-HETE by administration of an

effective amount of a 12(S)-HETE receptor antagonist.

10   The method is useful for the treatment or prophylaxis

of conditions in which 12(S)-HETE receptor activation

contributes to adverse effects. For example, the

method of this invention may be employed for the

treatment or prophylaxis of atherosclerotic

15   cardiovascular disease, glucose-induced complications

of diabetes, cytokine-induced inflammatory diseases and

tumor cell growth and metastasis.

The 12(S)-HETE receptor antagonist may be any

agent that blocks or significantly inhibits binding of

20   12(S)-HETE to its receptor. Such agents include DuP654

(2-phenylmethyl-1-napthol), Losartan (2-N-butyl-4-

chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-

yl)biphenyl-4-yl)methyl]imidazole, potassium salt),

pertussis toxin, 12(S)-HETE analogs, peptides and

20

peptide analogs having affinity for the binding site on
the 12(S)-HETE receptor (especially antibodies which
can block 12(S)-HETE receptors), antibodies to the
12(S)-HETE receptor, and the like.

5   The determination of appropriate, well-tolerated
dosage forms for administration to humans for use in
the present invention is within the ordinary skill in
the art. Such dosage forms include tablets, capsules,
syrups, suspensions, drops, injectable solutions,

10   lozenges, implants, transdermal patches, and other
dosage forms well known in the art for enteral or
parenteral administration. Based on _in vitro_
experiments on the effect of 12(S)-HETE blocking drugs
on 12(S)-HETE binding, a dose of between about 0.5 and

15   about 30 mg/kg/day would be effective in blocking
12(S)-HETE receptors in humans _in vivo_, and preferably
from about 1 to about 10 mg/kg/day.

        The present invention is further illustrated by
the following examples, which are not intended to be

20   limiting.

EXAMPLES

Example 1

Kinetics of [³H]12(S)-HETE binding to CHO-AT$_{1a}$

cells. Figures 1 and 2 are a competition curves which

5   examine the specificity of [³H]12(S)-HETE binding. CHO-

AT$_{1a}$ cells were grown to confluence in 24 well tissue

culture dishes in HAM's F12 medium containing 10% fetal

calf serum. The cells were then rinsed and placed in

fresh medium, HAM's F12/HEPES with no other additives

10   (450 ul per well). Serial dilutions of unlabeled

12(S)-HETE or DuP654 were added to the wells.

Commercial unlabeled 12(S)-HETE (BioMol Corp.) was

dried and reconstituted in ethanol to obtain a stock

solution of 5 mM. DuP654 was dissolved in DMSO to get

15   a stock solution of 5 mM. These were then serially

diluted and added in a volume of 1 ul to the wells to

obtain the final concentrations indicated. Then

[³H]12(S)-HETE (10,000 cpm in a volume of 50 ul per

well) was added from a stock solution obtained by

20   adding the tracer to the medium. The plates were then

incubated at 4°C with continuous shaking for 2 hr. The

cells were then washed 2 times with cold PBS and lysed

in 0.3N NaOH (200 ul). Radioactivity in the cell

lysates was quantitated in scintillation counter.

Affinities and binding constants were obtained using

Matlab computer software (Mlab, Civilized Software

Inc., Bethesda, MD).

This experiment revealed the presence of specific

5   high affinity binding sites for 12(S)-HETE on these

cells. A one site fit model yielded a Kd of 38.4 nM.

Specificity was determined by the observation that this

binding of [^{3}H]12(S)-HETE was displaced by unlabeled

12(S)-HETE.

10   Example 2

Reduction of cell growth induced by AII or 12(S)-

HETE.

DuP654 significantly reduced cell growth induced

by either AII or 12(S)-HETE at a concentration of 0.1

15   µM. Complete inhibition of 12(S)-HETE induced

mitogenic effects was seen. See Figure 3.

                              Example 3

Blockade of the 12(S)-HETE receptor by a specific

antagonist.

20   Tritrated 12(S)-HETE binding is blocked by

unlabeled 12(S)-HETE. See Figure 4. DuP654, a 12(S)-

HETE receptor antagonist, was shown also to block

12(S)-HETE at a concentration of 0.1 µM in both $AT_{1a}\Sigma$

and $AT_{1a}27$ cell types, two clones of CHO cells which

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overexpress the AII$_{1a}$ receptor. See Figure 4. The
cells were grown as described in Example 1.

Example 4

Blockade of 12(S)-HETE binding by pertussis toxin.

5   Cells were grown as described in Example 1. Prior to

addition of drug (12(S)-HETE or DuP654) to the cells,

the cultures were preincubated in HAM's F12 medium +

0.1% BSA for two hours at 37°C with or without 100

ng/ml pertussis toxin. Serial dilutions of unlabeled

10   12(S)-HETE or DuP654 were added, followed by [^{3}H]12(S)-

HETE as described for Example 1. After incubation and

washing, radioactivity in the cell lysates was

quantitated. See Figure 4. As discussed above,

pertussis toxin could also ablate 12(S)-HETE-induced

15   mitogenic effects. This implicates the involvement of

a G$_i$-protein-coupled receptor.

Example 5

Partial blockade of 12(S)-HETE mitogensis by the

specific angiotensin AT$_{1a}$ receptor antagonist, Losartan,

20   in CHO-AT$_{1a}$ cells. (Figure 5)

CHO-AT$_{1a}$ cells were plated in 12-well dishes (about

5-10,000 cells per well) for 24 hr. in growth medium

consisting of HAM's, F12 + 10% FCS. They were then

serum depleted for 72 hours by replacing the medium

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with HAM's F12 + 0.1% BSA. This medium was then

freshly replaced along with AII or 12-HETE (0.1 μM

each) prior to addition of drug to the cells. Losartan

was added as a solution in water to the cells 15 min.

5   prior to the addition of AII or 12-HETE. The final

concentration of Losartan was as indicated in Figure 4.

Fresh medium containing the same concentrations of AII

or 12(S)-HETE plus Losartan was replaced every 48

hours. At the end of 8 days, the medium was removed,

10   and 1 ml trypsin was added per well followed by 1 ml

isoton after 3 min. These trypsinized cells were

counted on a Coulter counter. Losartan partially

blocked 12(S)-HETE-induced mitogenic effects and fully

blocked AII-induced proliferative effects. See Figure

15   5). Losartan also partially blocked [^{3}H] 12-HETE binding

(Figure 4).

Example 6

Dependency of 12(S)-HETE mitogenic effects on

expression of the AT_{1a} receptor.

20   The three cell lines, CHO-AT_{1a}, CHO-AT_{1b} and mock

transfected CHO cells were gifts from Dr. Eric Clauser

(Inserm Unit, Paris, France). These cells were plated

in 12 well dishes in HAM's F12 medium + 10% FCS. After

72 hours serum depletion in HAM's F12 + 0.05% FCS, the

25

cells were treated with AII or 12(S)-HETE (0.1 µM).

Cells counts (after trypsinization) were taken at 48

hour intervals and fresh medium along with AII or

12-HETE added at these 48 hour intervals. 12(S)-HETE

5   had mitogenic effects only in CHO-AT$_{1a}$ cells, but not in

mock transfected cells (pSVneo), nor in CHO cells

overexpressing the angiotensin AT$_{1b}$ receptor. See

Figure 6.

Example 7

10   PAK activation by 12(S)-HETE.

CHO-AT$_{1a}$ cells were gently washed and placed in

depletion medium (HAM's F-12 medium containing 1 mg\ml

BSA and 20 mM HEPES, ph 7.4) for 72 hours prior to use.

After incubation for 30 minutes, the cells were treated

15   with 10^{-7}M 12(S)-HETE or with ethanol. The 12(S)-HETE

treatment was terminated by washing twice with PBS and

adding 300 µl lysis buffer (50 mM HEPES. pH 7.5,

containing 150 mM NaCl, 5 mM MgCl$_2$, 1 mM EGTA, 50 mM

NaF, 10 mM sodium pyrophosphate, 1% NP-40, 2.5%

20   glycerol and 1 mM Na$_3$VO$_4$ containing the protease

inhibitors phenylmethylsulfonyl fluoride, leupeptin,

and aprotonin) followed by sedimentation at 14,000 xg

at 4 °C for ten minutes. Protein determination was

performed by the Bradford method. The top panel shows

a representative autoradiogram of phosphonycated myelin

basic protein (MBP) bands from a gel. PAK activity was

measured as follows. First, 300 µg of lysate protein

was incubated with PAK antibody (1:20) in lysis buffer

5   overnight at 4°C, followed by incubation with 60 µl of

a 50% slurry of protein A beads for 60 minutes. After

washing three times with lysis buffer and twice with

kinase buffer (50mM HEPES, pH 7.4, 10mM $MgCl_2$, 10mM

$MnCl_2$, and 0.2 dithiothreitol) containing 2 µl MBP, 20

10   µM ATP and 5µCi [$\gamma-^{32}P$] ATP, the kinase activity was

measured in 60 µl kinase buffer. After incubation for

30 minutes at 30°C, the reaction was stopped with 5x

Laemmli sample buffer and resolved on a 12% SDS-

polyacrylamide gel, followed by autoradiography. The

15   bottom panel shows the densitometric quantitation of

PAK activity stimulated with 10^{-7} M 12(S)-HETE or

ethanol (control) for the time indicated. Each point

is an average (mean ± SE) from at least 3 separate

experiments. Results are expressed as stimulation over

20   control.

Example 8

Inhibition of 12(S)-HETE induced PAK activation by

transient transaction by a PAK binding domain (PDB)

plasmid.

The degree of 12(S)-HETE induce PAK activation was
compared in CHO-AT$_{1a}$ cells which had been transiently
transfected with a PDB plasmid and cells which had not
been transfected.

5   For the PBD-transfected group, CHO-AT$_{1a}$ cells were
transiently transfected with 15 μg PBD plasmid. For the
non-PBD-transfected group, CHO-AT$_{1a}$ cells were treated
with the same transfection reagents as the PBD-
transfected group, but lacking plasmid. Plasmids used

10   were endotoxin-free and prepared by EndoFree plasmid
kit (Qiagen Co.) with the standard protocol. The DNA
transfection method used was a cationic liposome-
mediated transfection with DOSPER transfection reagent
(Boehringer Manahein Co.) following the manufacturer's

15   instructions. Briefly, the cells were plated the day
before the transfection experiment at $3x10^6$ cells per
100 mm dish. The next day, cells were washed with
Opti-MEMd® reduced serum medium (Gibco BRL) and
incubated in 5 ml of HAM's F-12 medium containing 1%

20   FBS. Plasmid mixture (45 μg DUSPER/15 μg) was prepared
and added to each dish. After a 5 hour incubation, the
transfection medium was replaced and 8 ml fresh
depletion medium (described in Example 7) continuing 1%
FBS for overnight incubation was added. The cells were

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washed twice with depletion medium, incubated in the

same medium for another 32 hours and harvested. Cells

were then treated with 10⁻⁷M 12(S)-HETE or ethanol for

10 minutes. The top panel of Figure 8 illustrates a

5   representative autoradiogram of phosphorylated MBP

bands from a gel. The bottom panel illustrates the

densitometric quantification. Each point is an average

(mean ± SE) of at least 3 separate experiments.

Results are expressed as stimulation over control. The

10   PAK activity was measured as described in Example 7.

Example 9

Inhibition of PI 3-kinase by LY294002.

Cells were pretreated with different

concentrations of the PI-3 kinase inhibitor, LY294002

15   or DMSO (control) for 30 minutes, then treated with 10⁻⁷

M 12(S)-HETE or ethanol (control) for 10 minutes. PAK

activity was measured as described in Example 7.

Figure 9 shows a representative autoradiogram of

phosphorylated MBP bands from 3 similar experiments.

20   ## Example 10

Increased monocyte adhesion to HAEC by treatment

of monocytes with 12(S)-HETE.

Monocytes were incubated with 10-⁹M 12(S)-HETE for

12 minutes at room temperature (RT) or 37°C or left

untreated at room temperature and assayed for monocytes

adhesion.   Eight fields were counted for each

experiment.   Results are presented in Table 1.

Table 1 effect of 12(S)-HETE on Monocyte Adhesion to
Human Aortic Endothelial Cells

Experiment	NT	12(S)-HETE (RT)	12(S)-HETE (37°C)
1	26.4 ± 12.5	39.6 ± 12.5^{1}	39.0 ± 9.3^{3}
2	28.3 ± 5.0	45.4 ± 7.6^{2}	ND^{4}

1. $P = 0.016$
2. $P = 0.001$
3. $P = 0.01$
4. ND = not done

CLAIMS

1. A method for inhibiting the effects of 12-HETE comprising administration of an effective amount of a 12(S)-HETE receptor blocker.

2. The method of claim 1 wherein the receptors are on the cell surface.

3. The method of claim 2 wherein the in the cells are selected from the group consisting of monocytes, endothelial cells, pancreatic islet beta cells, nerve cells, cardiac fibroblasts, cardiac
5   myocytes and vascular smooth muscle cells.

4. The method of claim 1 wherein the administration of the compound is to an animal.

5. The method of claim 4 wherein the animal is a mammal.

6. The method of claim 4 wherein the animal is a human.

7. The method of claim 1 wherein the blocking of the receptor inhibits 12(S)-HETE binding.

8. The method of claim 1 wherein the blocking of the receptor inhibits receptor activation.

9. The method of claim 1 wherein the blocking of the receptor inhibits cell growth.

10.   The method of claim 1 wherein the blocking of the receptor inhibits inflammatory cell damage.

11.   The method of claim 1 wherein the blocking of the receptor inhibits cell death.

12.   The method of claim 1 wherein the blocking of the receptor reduces monocyte adhesion.

13.   The method of claim 1 wherein the blocking of the receptor reduces VEGF producion.

14.   The method of claim 1 wherein the blocking of the receptor reduces PAK activation.

15.   The method of claim 1 wherein the 12(S)-HETE receptor blocker is a 12(S)-HETE receptor antagonist.

16.   The method of claim 1 wherein the 12(S)-HETE receptor blocker is an antibody.

17.   A method of suppressing the activation of 12(S)-HETE receptors comprising the administration of a compound which prevents the binding of endogenous receptor agonists to the receptor.

18.   A method for the treatment or prophylaxis of a disease in which 12(S)-HETE receptor activation contributes to adverse effects which comprises administering an effective amount of a 12(S)-HETE receptor blocker.

19. The method of claim 18, wherein the disease

is atherosclerotic cardiovascular disease, glucose-

induced complications of diabetes, Parkinson's disease,

Alzheimer's disease, stroke-induced nerve damage,

5   cytokine induced inflammatory disease or tumor cell

growth or metastasis.

20. The method of claim 19, wherein the 12-HETE

receptor blocker is selected from the group consisting

of DuP654, Losartan, pertussis toxin, a 12(S)-HETE

analog, an antibody to the 12(S)-HETE receptor, a

5   peptide which binds to the 12(S)-HETE receptor and a

peptide analog which binds to the 12(S)-HETE receptor.

21. The method of claim 18, wherein the 12-HETE

receptor blocker is administered in a dose of from

about 0.5 to about 300 mg/kg/day.

22. The method of claim 21, wherein the 12-HETE

receptor blocker is administered in a dose of from

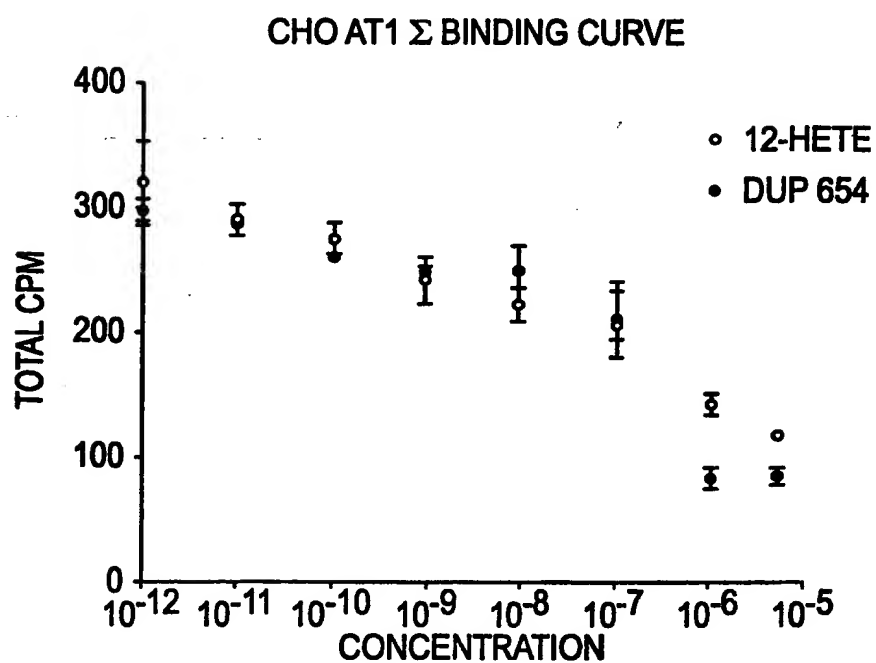about 1 to about 10 mg/kg/day.

FIG. 1

[³H]12(S)-HETE BINDING TO CHO-AT$_{1a}$ CELLS

CHO AT1 Σ BINDING CURVE

FIG. 2

COMPETITION OF COLD 12(S)-HETE FOR
12(S)-[³H]HETE BINDING TO CHO-AT₁ₐ CELLS
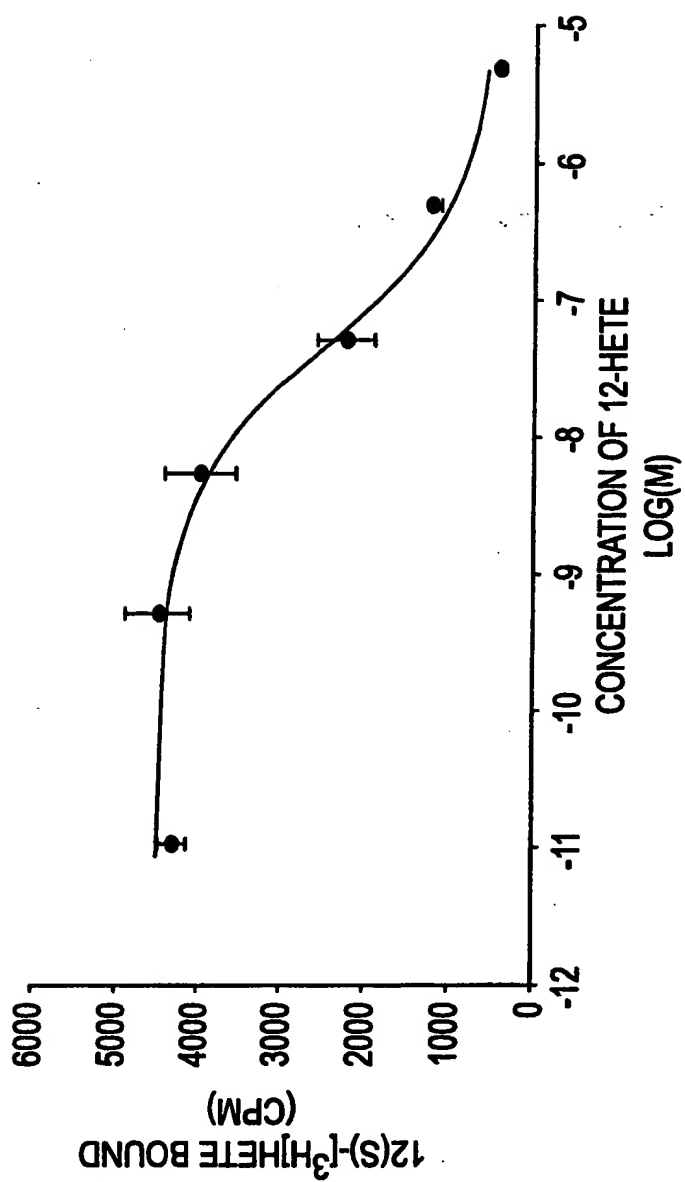
FIG. 3

EFFECT OF DuP654(12-HETE RECEPTOR ANTAGONIST) ON ALL AND 12-HETE-INDUCED GROWTH IN CHO-AT$_{1a}$ CELLS

* $p<0.001$ VS CONTROL
** $p<0.01$ VS ALL
+ $p<0.001$ VS CONTROL
++ $p<0.001$ VS 12-HETE
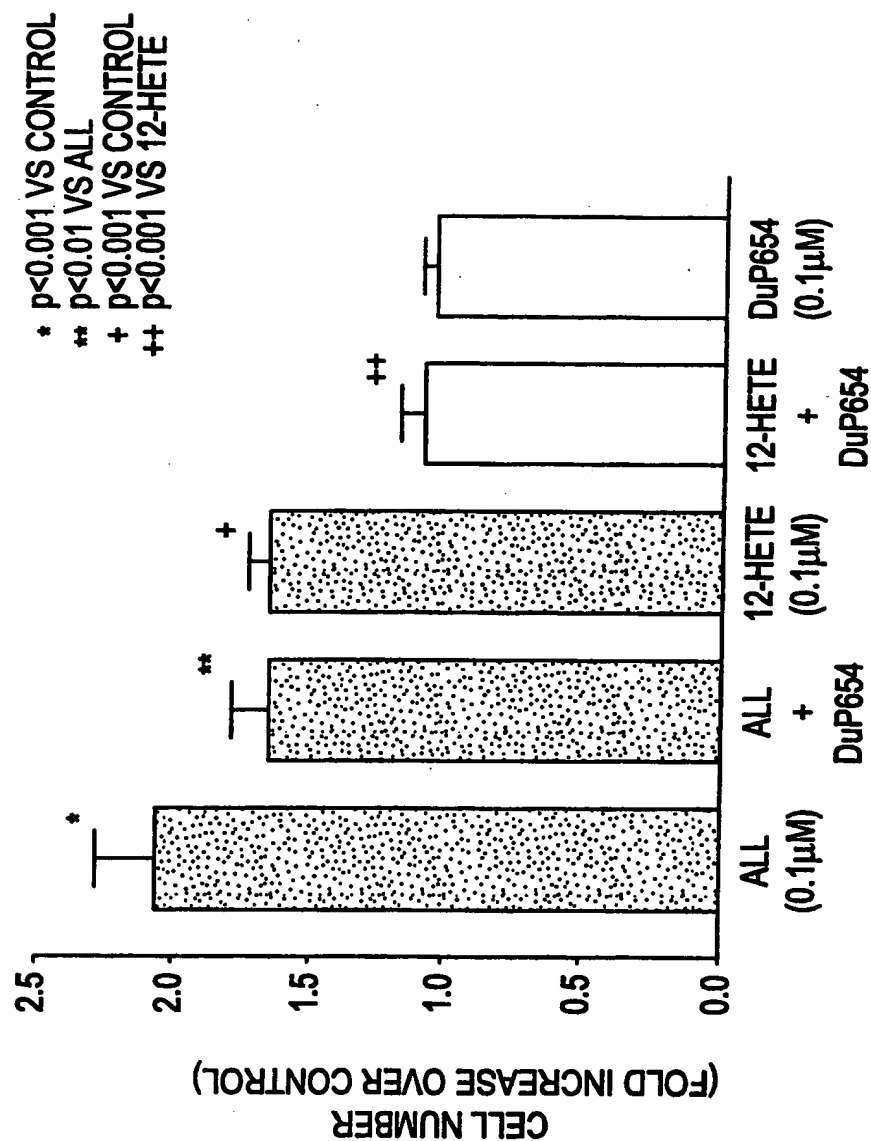
FIG. 4

EFFECT OF AGENTS ON 12(S)-[^{3}H] HETE
BINDING TO CHO-AT$_{1a}$ CELLS
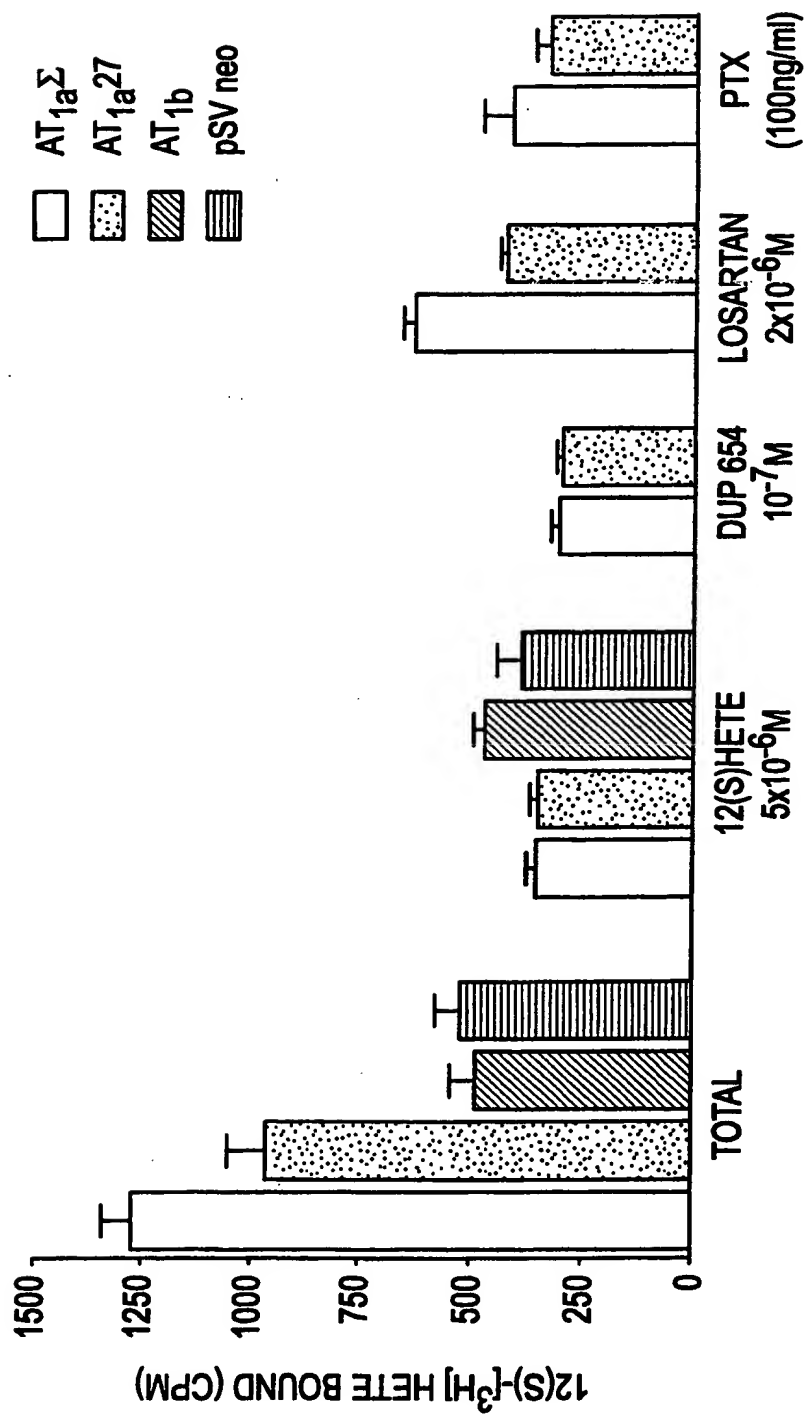
FIG. 5

EFFECT OF LOSARTAN(Los) ON ALL AND 12-HETE-INDUCED MITOGENESIS IN CHO-AT$_{1a}$ CELLS

*   p<0.001 VS CONTROL
**   p<0.001 VS ALL
+   p<0.001 VS CONTROL
++   p<0.01 VS 12-HETE
+++ p<0.001 VS 12-HETE

FIG. 6

FIG. 7

FIG. 8

CHO
GROWTH CURVE
0.05% BSA

○ CTRL
● ALL 10^{-7}M
▽ 12-HETE 10^{-7}M

FIG. 9

← MBP

* P<0.01 VS CONTROL
** P<0.001 VS CONTROL
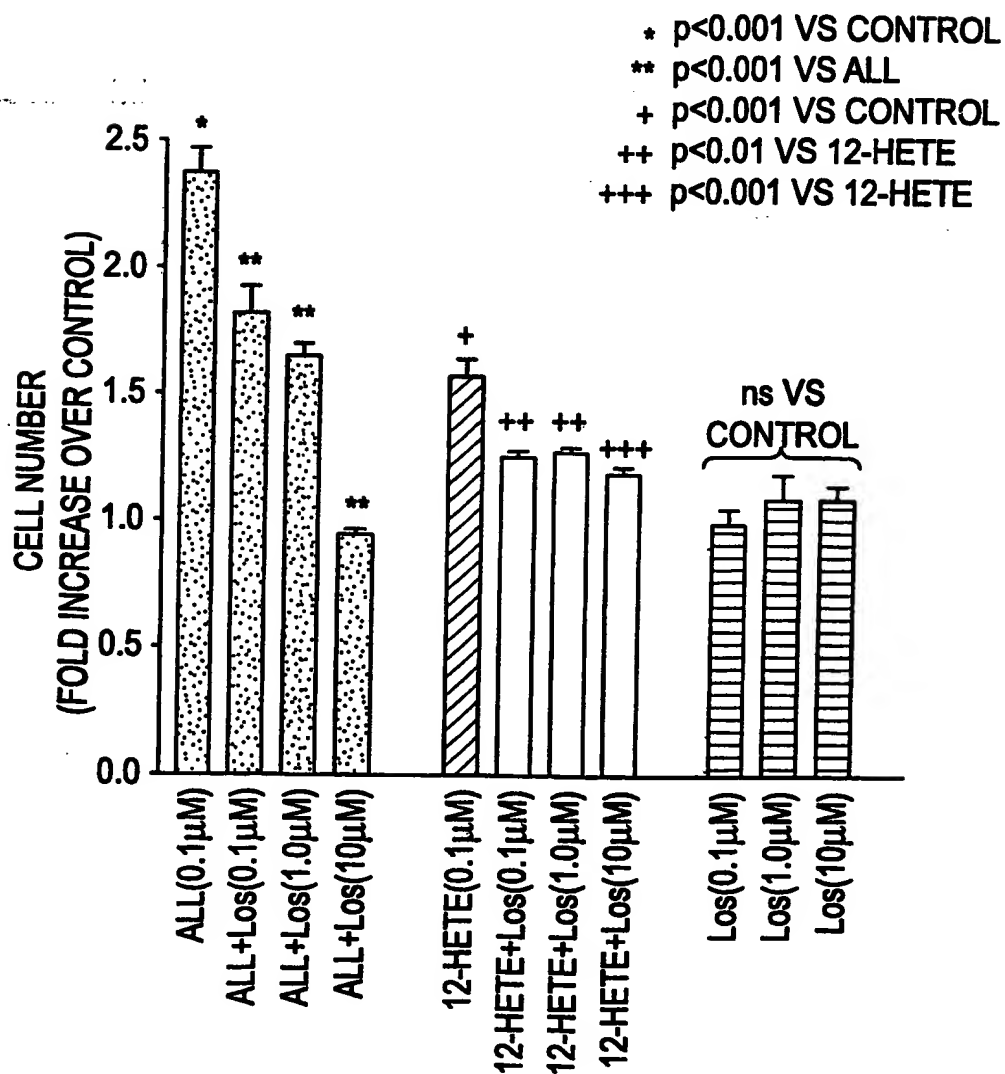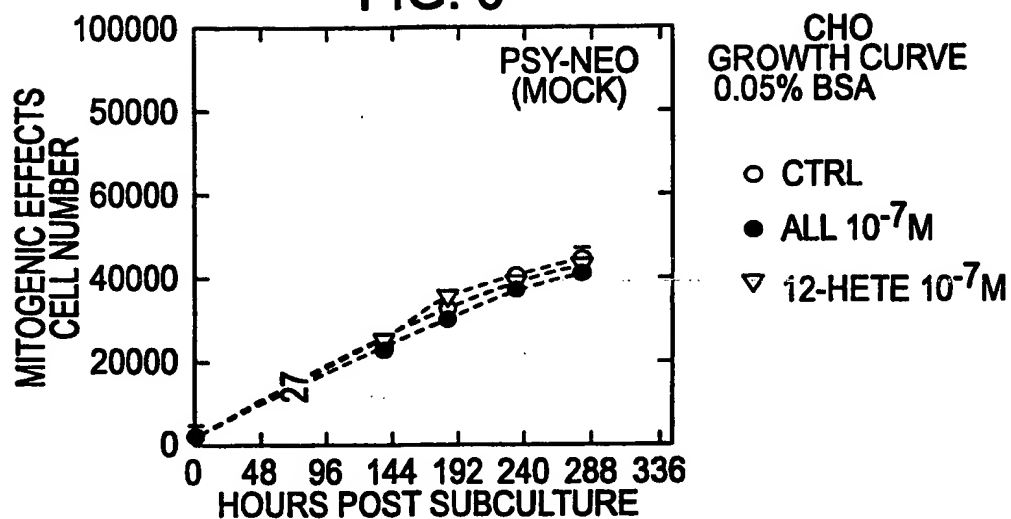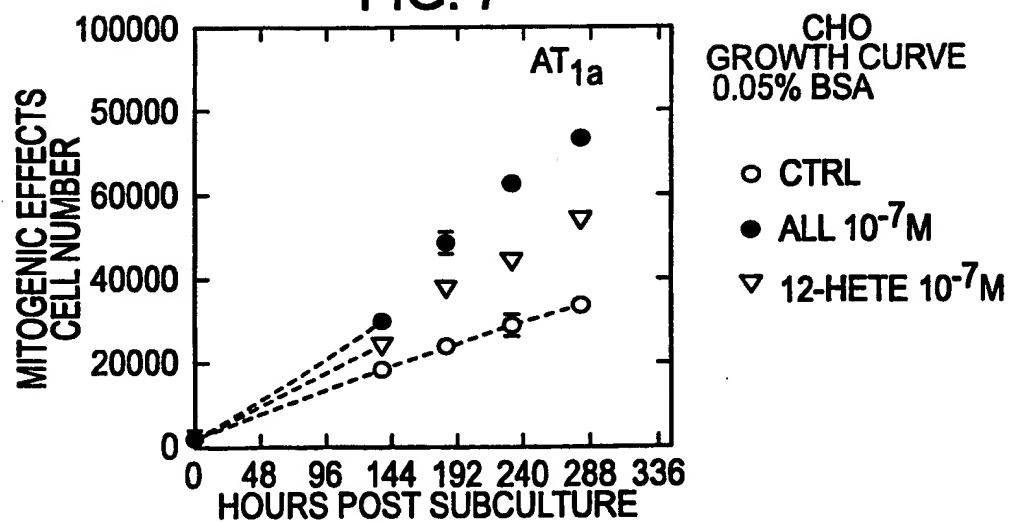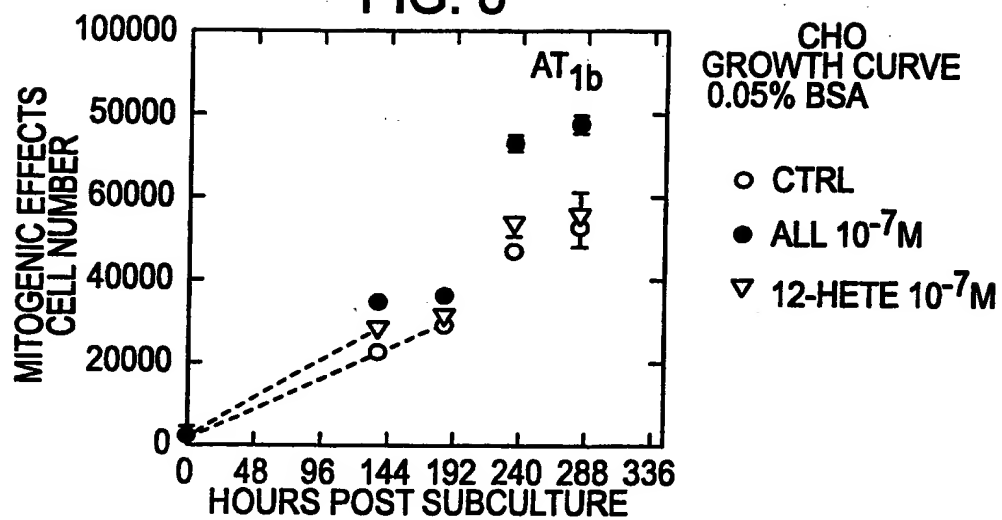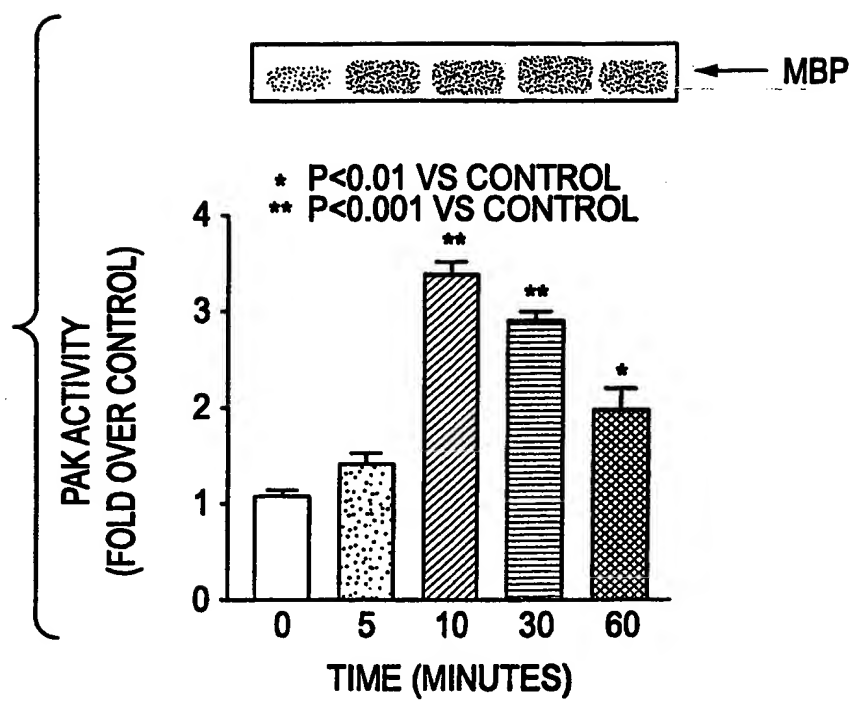
FIG. 10

FIG. 11

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   A61K31/415   A61K38/16   A61K31/20   A61K39/395   A61K31/05

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 34943 A (HOPE CITY ;NADLER JERRY L (US); NATARAJAN RAMA (US)) 7 November 1996 see page 3, line 28 – page 5, line 29 see page 23, line 9 – page 24, line 29 see page 26, line 32 – page 27, line 27 see page 31, line 24 – line 27 see page 32, line 10 – line 16 see page 43, line 20 – line 22 see page 49, line 33 – line 34 see claims 6,21,25,29–31 —/--	1-22

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 1999	02/03/1999

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Stein, A

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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ARENBERGER, PETR ET AL: "The lipoxygenase inhibitor 2-phenylmethyl-1-naphthol (DuP 654) is a 12(S)-hydroxyeicosatetraenoic acid receptor antagonist in the human epidermal cell line SCL-II" SKIN PHARMACOL. (1993), 6(2), 148-51 , XP002093788 cited in the application see the whole document	1,2, 4-10,15, 17-22
Y		3,11
Y	BLEICH D ET AL: "The stress-activated c-Jun protein kinase (JNK) is stimulated by lipoxygenase pathway product 12-HETE in RIN m5F cells" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATION, vol. 230, 1997, pages 448-451, XP002093789 cited in the application see the whole document	3,11
X	WEN Y ET AL: "Mechanisms of ANGII-induced mitogenic responses: role of 12-lipoxygenase and biphasic MAP kinase" AM.J.PHYSIOLOGY:CELL PHYSIOLOGY, vol. 40, no. 4, October 1996, pages c1212-c1220, XP002093790 cited in the application see the whole document	1-9,14, 18-22
A	NATARAJAN R ET AL: "MECHANISM OF ANGIOTENSIN II-INDUCED PROLIFERATION IN BOVINE ADRENOCORTICAL CELLS" ENDOCRINOLOGY, vol. 131, no. 3, September 1992, pages 1174-1180, XP000601236 see the whole document	1-15,21, 22

4

INTERNATIONAL SEARCH REPORT

B x I   Obs rvations where certain laims w re f und uns archabl (C ntinuation of item 1 f first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority. namely:

   Remark: Although claim(s) 1-22
           is(are) directed to a method of treatment of the human/animal
           body, the search has been carried out and based on the alleged
           effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of Invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application. as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all
   searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee. this Authority did not invite payment
   of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant. this International Search Report
   covers only those claims for which fees were paid. specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently. this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                               [ ] No protest accompanied the payment of additional search fees.

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Patent document cited in search report		Publication date	Patent family m mber(s)		Publication date
WO 9634943	A	07-11-1996	AU	5637796 A	21-11-1996
			CA	2220156 A	07-11-1996
			EP	0824583 A	25-02-1998